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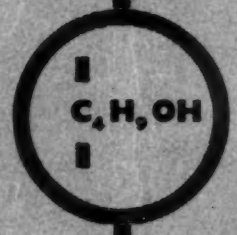

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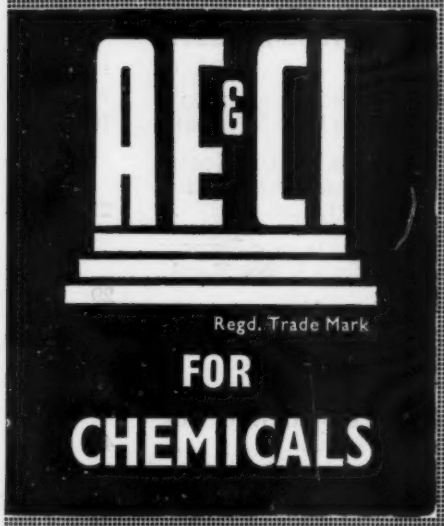

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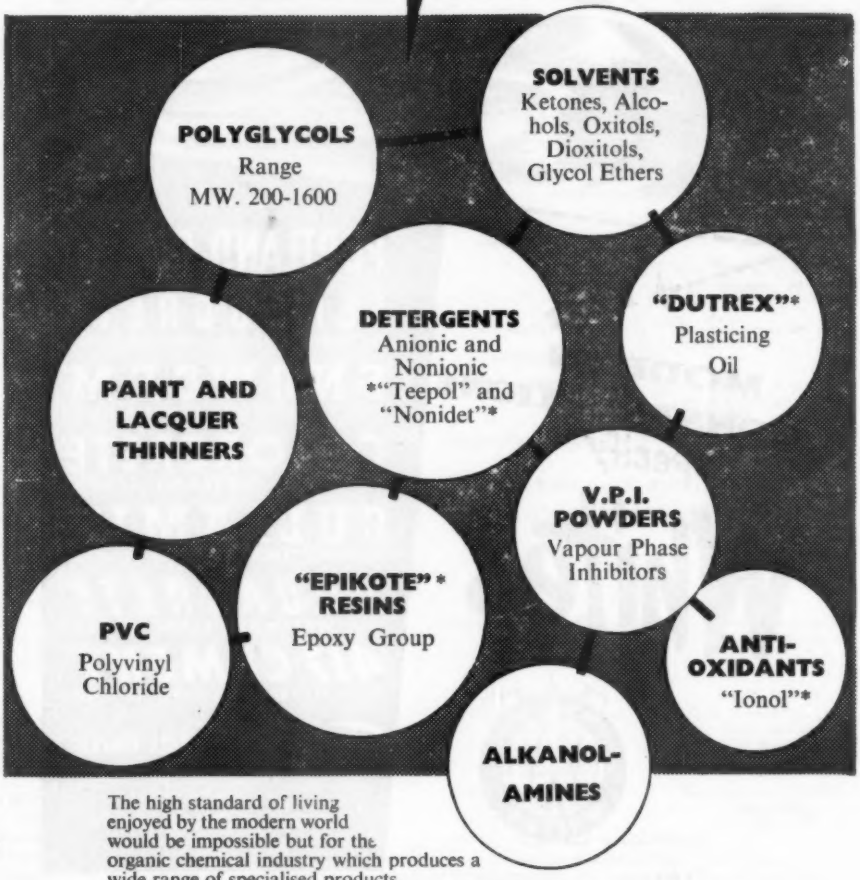

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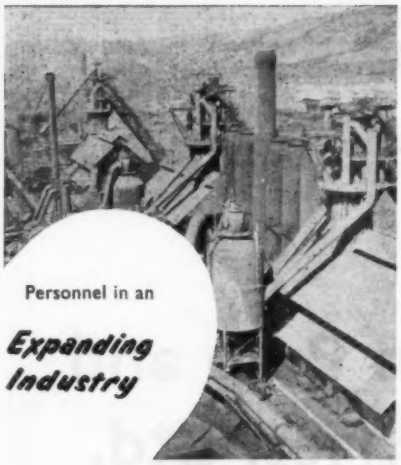

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A PRELIMINARY STUDY OF THE INFRA-RED ABSORPTION SPECTRA OF THE *SENECIO* ALKALOIDS

by

E. C. LEISEGANG* and B. O. G. SCHÜLER†

OPSOMMING

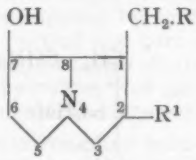
'n Interpretasie van die infrarooi spektra van 'n aantal *Senecio*alkaloiede en *necine*base word gegee. Die ondersoeking van oplossings in koolstoftetrachloried lei tot 'n nadere bepaling van die molekulêre strukture en lewer resultate wat ooreenstem met dié van ander werkers.

SUMMARY

An account is given of the interpretation of the infra-red spectra of a number of *Senecio* alkaloids and *necine* bases. Studies of the carbon tetrachloride solutions lead to a closer elucidation, in agreement with the work of others, of molecular structure.

Alkaline hydrolysis of those *Senecio* alkaloids occurring in plant material of local origin[1], yields a number of *necic* acids and *necine* bases. The bases form a graded series of hydroxylated derivatives of pyrrolizidine, suitable for spectroscopic study. The known structures[1] of five of these derivatives are given in Table I which also lists the wave numbers of the broad infra-red absorption bands assigned to the hydroxyl groups of crystalline samples of the compounds.

TABLE I



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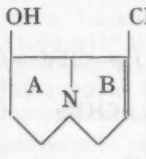


II

		O-H WAVE NUMBER	
Retronecanol	I, R=R[1]=H	—	3,350
Platynecine	I, R=OH, R[1]=H	—	3,340
Rosmarinecine	I, R=R[1]=OH	3,176	3,320
Retronecine	II	—	3,320
Isatinecine	II : N-oxide	3,100	—

With the exception of isatinecine, the integrated intensities of the bands between 3,100 and 3,500 cm.$^{-1}$, relative to the intensities of the C-H stretching fundamentals (2,500 to 3,000 cm.$^{-1}$), were found to provide a rough measure of the number of hydroxyl groups per molecule. The 3,176 cm.$^{-1}$ band of rosmarinecine would seem to be due to the hydroxyl group in the 2-position. Isatinecine differs markedly from the other derivatives listed in that it is an N-oxide[2]. The substance solidifies without water of crystallisation and the spectrum therefore indicates[3] that the solid might well exist in the form of an intermolecular hydrogen bridge chelation complex.

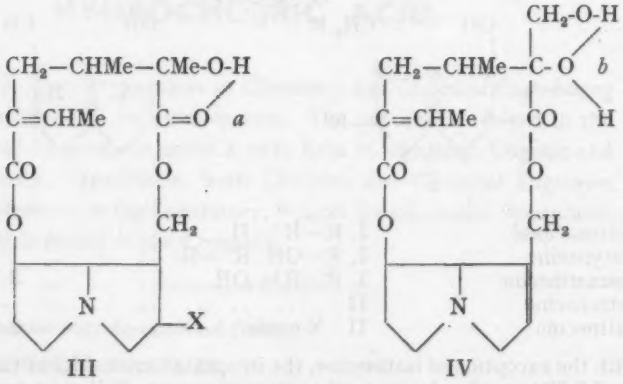
**Present Address:* 26, Elm Avenue, P.O. Northrand, Transvaal.
†*Present Address:* Magdalene College, Cambridge, England.

Purified carbon tetrachloride proved the most suitable spectroscopic solvent for the detailed study of the hydroxy stretching fundamentals of three of the bases; isatinecine and rosmarinecine were, however, insoluble in non-polar solvents. The solutions of retronecanol, platynecine and retronecine all showed infra-red absorption bands at 3,630 and at 3,325 cm.$^{-1}$. The extinction coefficients of the strong sharp bands at the higher frequency varied inversely with the concentrations of the solutions and the bands were thus characteristic[3, 4, 5] of "free" hydroxy groups. On the other hand, the extinction coefficients of the broad bands of lower frequency varied directly with concentration and the bands were therefore indicative[3, 4, 5] of intermolecular hydrogen bridge formation. In agreement with the known structures, therefore, the three soluble bases have "free" hydroxy groups when dispersed, but form intermolecular hydrogen bridges in concentrated solution and in the solid phase.

Platynecine and retronecine solutions further show fairly sharp bands at 3,467 and 3,515 cm.$^{-1}$ respectively which obey the Beer-Lambert law. These bands are characteristic[3, 4, 5] of intramolecular hydrogen bridge structures which can only exist, as shown by a study of the molecular models, if both hydroxy groups lie on the same side of the molecule, within the fold and directed away from the nitrogen lone pair orbital. These observations thus support previous conclusions on the stereochemistry of the *necine* bases[6]. Retronecine differs from platynecine in that the 1:2-double bond and the central atom of the -CH_2.OH group must necessarily lie in the plane of ring B. The strained intramolecular hydrogen bond of retronecine would be expected to relieve the tension on the O-H bond which would thus be shorter and stronger than that of platynecine. The higher frequency in the spectrum of retronecine is thus accounted for stereochemically.

The *Senecio* alkaloids are esters, four of which are formulated[1] in Table II.

TABLE II



		O-H Wave Number (Solid)	
Platyphylline	III, X=H	3,440	3,590
Rosmarinine	III, X=OH	3,410	—
Retrorsine	IV	—	3,590
Isatidine	IV:N-oxide,2H_2O ..	3,440	—

The 3μ spectrum of isatidine, like that of isatinecine, is unique. Isatidine is an N-oxide which solidifies with water of crystallisation. The absence of a "free" hydroxy

chromophore for solid rosmarinine might be due to hydrogen bond formation connected with the hydroxy group X, structure III. The low frequency bands may be attributed to the existence in the solid compounds of hydrogen bonds; the absence of a band in the case of retrorsine might be accounted for in terms of its crystal structure.

Carbon tetrachloride again proved suitable as a spectroscopic solvent for these alkaloids, except isatidine. Platyphylline, rosmarinine and retrorsine solutions all showed a broad band at 3,420 cm.$^{-1}$, the extinction coefficient of which varied directly with concentration. This band was therefore due [3,4,5] to intermolecular hydrogen bond polymerisation. Dilute solutions of retrorsine gave evidence of "free" hydroxy groups in that a sharp band appeared at 3,603 cm.$^{-1}$.

The solutions of the alkaloids also showed moderately sharp bands, assigned to intramolecular hydrogen bridges, the frequencies of which indicated the existence of the two structural types, III*a* and IV*b*. For stereochemical reasons, the bridge at *a* suffers greater strain than that at *b*, in agreement with the lower frequency (3,512 cm.$^{-1}$) for retrorsine compared with those observed for platyphylline and rosmarinine (3,547 and 3,560 cm.$^{-1}$ respectively).

The intramolecular bond band of rosmarinine was about twice as intense as that shown by platyphylline. This is probably due to the hydroxy group X, structure III, contributing to the rosmarinine spectrum.

The spectra of the solid alkaloids all showed weak bands near 1,650 cm.$^{-1}$, assigned to the open-chain double bonds, and two strong sharp bands near 1,715 and 1,740 cm.$^{-1}$ due, respectively, to the conjugated and isolated carbonyl groups. The complete spectra for the nine compounds studied are available [7] at these laboratories.

EXPERIMENTAL

Pure specimens, as shown by their melting points [1,8] of rosmarinecine and platynecine were kindly supplied by Professor F. L. Warren. Isatidine, extracted from *S. isatideus* [9], was purified and part of the material reduced [10] to retrorsine. Platyphylline and rosmarinine were obtained from *S. Hygrophilus*.[8] Isatinecine was prepared by alkaline hydrolysis [11] of isatidine and separate portions of the product were reduced [10] to retronecine and retronecanol. Retronecine was crystallised from acetone.

The single-beam recording infra-red spectro-radiometer [11] constructed in these laboratories was used. A calcium fluoride prism was used for the 3 μ region and sodium chloride and potassium bromide prisms to cover the remainder of the spectrum up to 20 μ. Liquid paraffin pastes of the solid compounds were compressed between two optical plates and formed absorption cells of suitable thickness. Perfluoroparaffin pastes were used for the 3 μ region and over the range 1,350 to 1,500 cm.$^{-1}$. Potassium bromide optical plates were used beyond 14 μ.

An absorption cell, designed for the study of the carbon tetrachloride solutions, was made by fixing rock salt plates to the faces of a brass washer, 1 cm. thick, with *Secotine* adhesive. The solutions were admitted into the cavity of the cell *via* a small stoppered hole through the brass. The spectra of saturated solutions (*ca.*0.1 molar) of the samples were compared with those of a number of diluted solutions.

The authors acknowledge a bursary (to B.O.G.S.) and grants for equipment and a research assistant (to E.C.L.) from the South African Council for Scientific and Industrial Research.

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THE STRUCTURE OF THE ADDUCT OF BENZONITRILE OXIDE TO INDENE, AND THE SYNTHESIS OF 1- AND 2-BENZOYLINDANE

by

G. W. PEROLD AND F. V. K. VON REICHE

OPSOMMING

Die splitsing deur konstant-kokende joodwaterstofsuur van die 2-isoksasolien gevorm deur die addisie van bensonitrieloksied aan indeen lewer 2-bensoïel-indaan. Die addisie volg dus die Markownikoff reël om 3-feniel-4, 5-(indano-2′, 1′)-2-isoksasolien te vorm. Die aard van die neweprodukte van die splitsingsreaksie word bespreek.

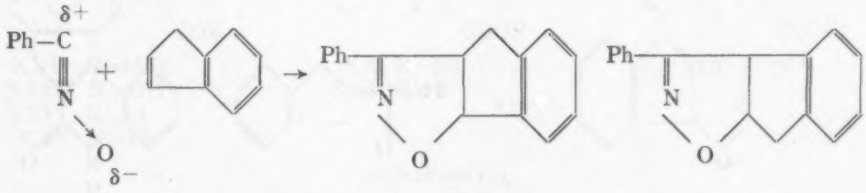
Die sintese van die totnogtoe onbekende 1- en 2-bensoïel-indane deur reaksie van difenielkadmium met die ooreenkomstige suurchloriede word beskryf.

SUMMARY

Refluxing constant-boiling hydriodic acid cleaves the 2-isoxazoline obtained by addition of benzonitrile oxide to indene, to 2-benzoyl-indane. The addition therefore follows the Markownikoff rule and leads to 3-phenyl-4, 5-(indano-2′, 1′) -2-isoxazoline. The nature of the by-products of the cleavage reaction is discussed.

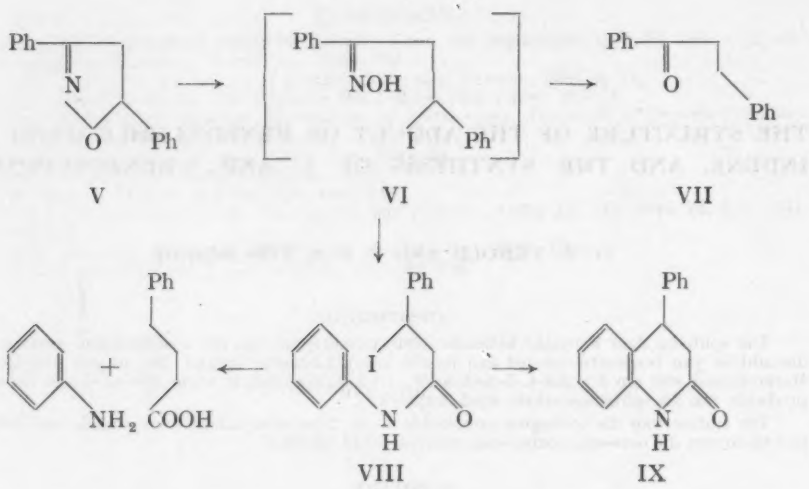
The synthesis of the hitherto unknown 1- and 2-benzoylindanes by the reaction of diphenylcadmium with the corresponding acid chlorides is described.

The addition of benzonitrile oxide[1] to aryl olefines[2] leads to the formation of 2-isoxazolines[3]. As benzonitrile oxide generally adds as a dipole, polarized as shown (I), its addition to indene (II)[4] would yield III following the Markownikoff rule, while structure IV would result from non-Markownikoff addition.



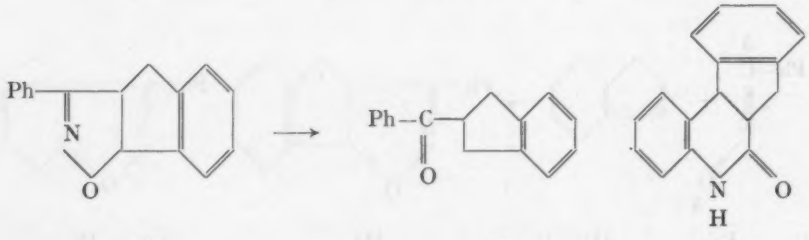
I II III IV

In a study[5] of the chemical behaviour of the 2-isoxazolines, we have shown that they react as cyclic O-alkyl oxime ethers under the conditions of the Zeisel determination of alkoxy groups. The main product obtained under these hydrolytic and reducing circumstances from, e.g., 3, 5-diphenyl-2-isoxazoline (V) is ω-benzyl-acetophenone (VII) *via* the intermediate iodo-oxime (VI). In this reaction, a Beckmann rearrangement of VI to the iodoanilide VIII also occurred, followed partly by hydrolysis and reductive dehalogenation to give dihydrocinnamic acid and aniline, and partly by cyclization with elimination of hydriodic acid to give 4-phenyl-3, 4-dihydrocarbostyril (IX).



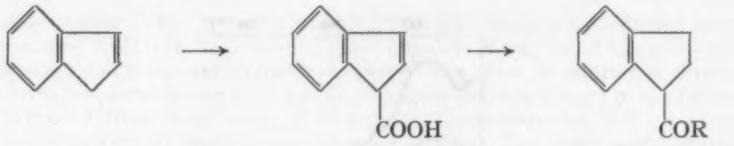
On applying the same cleavage conditions to the addition product obtained from benzonitrile oxide and indene (III or IV), the ketonic product was 2-benzoylindane (X). The addition product was therefore 3-phenyl-4, 5-(indano-2′,1′)-2- isoxazoline (III), formed in accordance with the Markownikoff rule.

As in the model experiments on 3, 5-diphenyl-2-isoxazoline (above), the main product was accompanied by acid products and by a high-melting neutral substance. The latter compound, $C_{16}H_{13}ON$, m.p. 269-270°, may in accordance with the formation of the carbostyril (IX) be formulated as 3, 4-(indano-2′, 1′)-3, 4-dihydrocarbostyril (XI). The physical data (see Experimental Section) are in accord with this formulation.



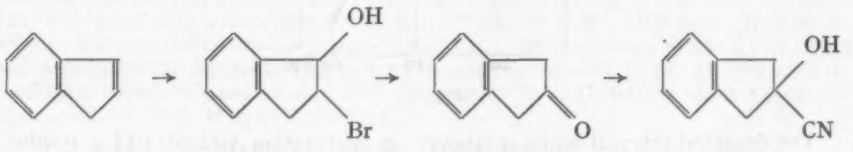
The reference substances required for the above structural derivation, *viz.* the 1- and 2- benzoyl derivatives of indane, were both unknown. A synthesis of 2-benzoylindane, reported by Perkin and Révay[6], was found to be erroneous. Their procedure, the direct Friedel-Crafts acylation of benzene with indane-2-carbonyl chloride, gave a product, m.p. 107°, which was not in any way characterized. In our hands their procedure yielded an amorphous and probably impure product, m.p. 135°, $(C_{12\cdot7}H_{11\cdot4}O)_n$, which gave no oxime or 2, 4-dinitrophenylhydrazone. The same reaction carried out with indane-1-carbonyl chloride similarly failed to give satisfactory results.

Smooth reaction was obtained by reacting the respective indane-carbonyl chlorides with diphenylcadmium[7]. 1-Benzoylindane (XV) was so obtained as a colourless liquid, which gave an oxime, m.p. 111°, and a semicarbazone, m.p. 210°. The ketone regenerated from the semicarbazone was still a liquid, b.p. 130-135° (bath)/1 mm.

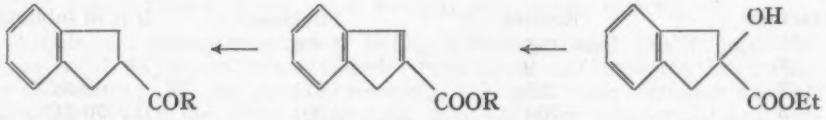


II XII XIII, R=OH
XIV, R=Cl
XV, R=Ph

2-Benzoylindane (XXV) was similarly obtained from indane-2-carboxylic acid (XXIII), which was here prepared by a new route. It melted at 70-71°, also when regenerated from its oxime (m.p. 129·5°), and also afforded a semicarbazone, m.p. 186-188°.



II XVI XVII XVIII



XIX XX, R=Et
XXI, R=H
XXII, R=OEt
XXIII, R=OH
XXIV, R=Cl
XXV, R=Ph

EXPERIMENTAL

All m.p.s. are corrected. Ultraviolet absorption measurements were carried out in ethanol using the Zeiss Opton M4Q spectrophotometer. Infrared spectra were taken in potassium bromide dispersions on the Perkin-Elmer Model 21 instrument.

The Reductive Cleavage of the Indene Adduct. The adduct (2·0 g.) of m.p. 134°, obtained[3] from benzonitrile oxide and indene, was refluxed for 1 hr. with constant boiling hydriodic acid (40 ml.) with liberation of free iodine. After dilution with water, the mixture was extracted with ether, a small precipitate (5 mg.) being discarded. The ethereal extract was decolourised with sodium thiosulphate solution and then washed with sodium hydrogen carbonate solution, thus removing 96 mg. acid products. On concentrating the neutral extract, a colourless crystalline precipitate formed (135 mg., mp. about 250°) which was recovered by decanting the

supernatant solution. Crystallisation from alcohol and from methyl ethyl ketone gave colourless prisms, m.p. 269-270°, probably XI; $\lambda_{max.}$ 254 mμ, log ε 4·05; the shape of the ultraviolet spectrum was the same as that of 4-phenyl-3,4-dihydro-carbostyril[5]. Amide NH and amide carbonyl absorption[5] occurred at 3190 and 1669 cm.$^{-1}$ respectively (Found: C, 81·85; H, 5·7. Calcd. for $C_{16}H_{13}ON$: C, 81·7; H, 5·6%).

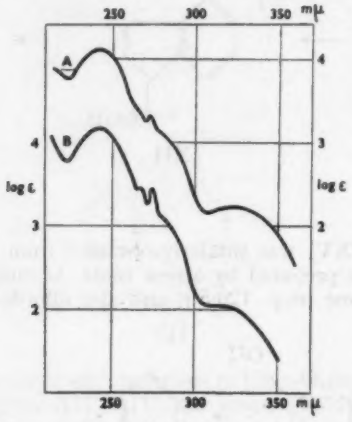


Fig. 1

The decanted ethereal solution (above) on evaporation yielded 1·14 g. residue which was dissolved in light petroleum and benzene (2:1.) (75 ml.), adsorbed on alumina (40g.) and eluted with the same solvent in 50 ml. fractions. The material eluted in each fraction was sublimed *in vacuo*.

Fraction	Residue mg.	Sublimate mg.	M.p. of sublimate
1	9	—	—
2	225	174	60-66°
3	354	294	70-71°
4	109	106	68-70°
5	35	31	60-65°
6	12	—	—

The sublimate obtained from fraction 3 was pure 2-benzoyl-indane, m.p. 70-71°, undepressed by the reference compound (below) ($\lambda_{max.}$ mμ/log ε: 243/4·20, 266/3·47, 274/3·44, 315/2·00 (shoulder)). The infrared absorption was at all points identical with that of the reference compound (Found: C, 86·5; H, 6·6. Calcd. for $C_{16}H_{14}O$: C, 86·45; H, 6·3%). Further amounts of 2-benzoyl-indane, m.p. 70°, were obtained by combining and rechromatographing fractions 2, 4 and 5. The *oxime*, obtained from the ketone (102 mg.) and hydroxylamine hydrochloride (500 mg.) by boiling for 4 hrs. in pyridine and ethanol (2:1), was crystallised from light petroleum; m.p. 129-130°, undepressed by the oxime, m.p. 129·5°, of synthetic 2-benzoyl-indane (below) (Found: C, 81·15; H, 6·5. Calcd. for $C_{16}H_{15}ON$: C, 81·0; H, 6·4%).

Indene-1-carboxylic acid (*XII*) was prepared by the carbonation of sodium-indene[8] in 38 *per cent.* yield and crystallised from benzene as yellowish needles, m.p. 156° (Found: C, 74·85; H, 5·2. Calcd. for $C_{10}H_8O_2$: C, 75·0; H, 5·0%).

Indane-1-carboxylic acid (*XIII*). Indene-1-carboxylic acid (30g.) in ethanol was hydrogenated over Adams' platinic oxide (0·25g.) until 1·05 mole hydrogen had been taken up. The filtered solution was evaporated and the residue crystallised from petroleum ether to give colourless prisms, m.p. 51-52°* (Found: C, 73·9; H, 6·3. Calcd. for $C_{10}H_{10}O_2$: C, 74·05; H, 6·2%).

1-*Benzoylindane* (*XV*). The acid chloride (XIV) was obtained by refluxing indane-1-carboxylic acid (XIII) (1·70 g.) with thionyl chloride (3 ml.) for 2 hr., evaporating the excess reagent *in vacuo*, adding benzene and drying again. A solution of diphenylcadmium[7] was prepared by carefully adding cadmium chloride (4·4 g.) to the Grignard solution prepared from magnesium (0·98 g.) and bromobenzene (6·6 g.) in ether (40 ml.) and then kept at room temperature for 30 mins. The crude acid chloride in dry benzene (50 ml.) was stirred under reflux and the diphenylcadmium solution added during 20 mins. After refluxing for a further 10 mins., the mixture was carefully poured into crushed ice and hydrochloric acid. The benzene layer was washed with dilute hydrochloric acid, sodium hydroxide and water, evaporated *in vacuo* and the residue distilled. The crude ketone (1·72 g., 74 *per cent.*) was obtained at 146°(bath)/0·5 mm. and directly converted to derivatives.

The *oxime* was obtained from crude XV (99 mg.) and hydroxylamine hydrochloride (150 mg.) in pyridine and ethanol as crystals (41 mg.), m.p. 108°, raised to 111° by crystallisation from light petroleum. (Found: N, 6·29. Calcd. for $C_{16}H_{15}ON$: N, 5·9%). The crystalline *semicarbazone* (0·85 g.) was obtained from crude XV (1·85 g.) and semicarbazide hydrochloride (1·3 g.) in aqueous alcohol (2 hr. at 75°) and recrystallised from ethanol to m.p. 210° (Found: C, 72·7; H, 6·0; N, 15·3. Calcd. for $C_{17}H_{17}ON_3$: C, 73·1; H, 6·1; N, 15·0%).

The *pure ketone* (XV) was regenerated from the semicarbazone (405 mg., m.p. 210°) by refluxing in 0·5N aqueous hydrochloric acid (80 ml.) for 3 hrs. The neutral product recovered by ether extraction (324 mg. liquid) distilled sharply at 130-135° (bath)/1 mm. as a colourless liquid. (282 mg.; $\lambda_{max.}$ mμ/logϵ: 243/4·12, 273/3·32, 325/2·22. Found: C, 86·3; H, 6·4. Calcd. for $C_{16}H_{14}O$: C, 86·45; H, 6·3%).

2-*Hydroxy-2-ethoxycarbonylindane* (XIX). 2-Bromo-indanol (XVI) (m.p. 124-125°, av. yield, 86 *per cent.*) was obtained[10] from indene and converted[11] to 2-indanone (XVII) (m.p. 55°, av. yield, 87 *per cent.*). This gave[12] the somewhat unstable cyanhydrin (XVIII) (av. yield, 75 *per cent.*, m.p. 123° from ethanol and light petroleum (Found: C, 75·2; H, 6·1. Calcd. for $C_{10}H_9ON$: C, 75·4; H, 5·7%). The crude cyanhydrin (2·16 g., m.p. 107°) was kept in 5 *per cent.* alcoholic hydrogen chloride (60 ml.) at room temperature for 66 hr. Concentration of the solution and addition of water gave colourless needles of XIX (2·15 g.), m.p. 90° unchanged on crystallisation from aqueous alcohol (Found: C, 69·6; H, 6·35. Calcd. for $C_{12}H_{14}O_3$: C, 69·9; H, 6·8%).

2-*Ethoxycarbonylindene* (XX). 2-Hydroxy-2-ethoxycarbonylindane (2·95 g.) in benzene (200 ml.) was treated with phosphorus pentoxide (10 g.) for 3 hr. at 80°. The decanted solution was washed with water, evaporated and the residue (1·82 g.) twice distilled at 93° (bath)/0·2 mm. to give colourless crystals, m.p. 48°† (av. yield, 55 *per cent.* Found: C, 76·4; H, 6·4. Calcd. for $C_{12}H_{12}O_2$: C, 76·55; H, 6·4%). Saponification of a small sample in aqueous potassium hydroxide gave indene-2-carboxylic acid (XXI), m.p. 232° (lit.[14] m.p. 234°) (Found: C, 74·7; H, 4·9. Calcd. for $C_{10}H_8O_2$: C, 75·0; H, 5·0%).

*Tiffeneau and Orechow[9] reported m.p. 59-60° for XIII obtained by sodium amalgam reduction of XII.

†von Auwers[13] found m.p. 50° for material prepared by a different route.

2-Ethoxycarbonylindane (XXII). 2-Ethoxycarbonylindene (1·45 g. in 75 ml. alcohol) was hydrogenated in the presence of palladium on barium sulphate (1·19 g., 2 *per cent.* palladium) and 5N hydrochloric acid (1 ml.), 0·90 mole hydrogen being taken up. Distillation of the product gave an essentially quantitative yield of XXII, b.p. 105° (bath), 0·5 mm. (Found: C 76·0; H, 7·7. Calcd. for $C_{12}H_{14}O_2$: C, 75·8; H, 7·4%).

Indane-2-carboxylic acid (XXIII). The foregoing ester (1·45 g.) was heated on the water bath for 90 mins. with potassium hydroxide (1·6 g.) in water (10 ml.) and the product recovered as the acid (1·25 g.), m.p. 127-128°, in quantitative yield. (Lit.[16] m.p. 130° *via* another route) (Found: C, 74·05; H, 6·5. Calcd. for $C_{10}H_{10}O_2$: C, 74·05; H, 6·2%).

2-Benzoylindane (XXV). Indane-2-carboxylic acid (1·25 g.) was converted to the acid chloride as before and this distilled at 97° (bath)/0·2 mm. in 76 *per cent.* yield. The acid chloride (1·15 g.) was treated with diphenylcadmium as before and yielded 1·44 g. (quantitative yield) of crude XXV, m.p. 60°. Crystallisation from alcohol gave the pure ketone, m.p. 70-71° (Found: C, 86·4; H, 6·4. Calcd. for $C_{16}H_{14}O$: C, 86·45; H, 6·3%). The *oxime* was obtained as above, m.p. 129·5°, from petroleum ether (Found: C, 81·4; H, 6·2. Calcd. for $C_{16}H_{15}ON$: C, 81·0; H, 6·4%). The *semicarbazone*, prepared as above, melted at 186-188° (Found: C, 72·9 H, 6·3. Calcd. for $C_{17}H_{17}ON_3$: C, 73·1; H, 6·1%). Pure *2-benzoylindane* was regenerated from the oxime as in the case of the semicarbazone of 1-benzoylindane. It was obtained in 87 *per cent.* yield, m.p. unchanged at 70-71° [λ_{max}.mμ/log ε: 243/4·15, 267/3·45, 274/3·43, 320/2·06 (shoulder). Found: C, 86·8; H, 6·4. Calcd. for $C_{16}H_{14}O$: C, 86·45; H, 6·3%].

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CONJUGATION EFFECTS IN THE CYCLOPROPANE SERIES. THE UNAMBIGUOUS SYNTHESIS AND PROPERTIES OF *TRANS*-2-PHENYL-3,3-DIMETHYLCYCLOPROPANECARBOXAMIDE

by

G. W. PEROLD

OPSOMMING

Die amied in die titel genoem is vanaf 2-diaso-propaan en etielsinnamaat sintetiseer en sy struktuur *via* 'n afbou met osoon na *trans*-karoonsuur bevestig.

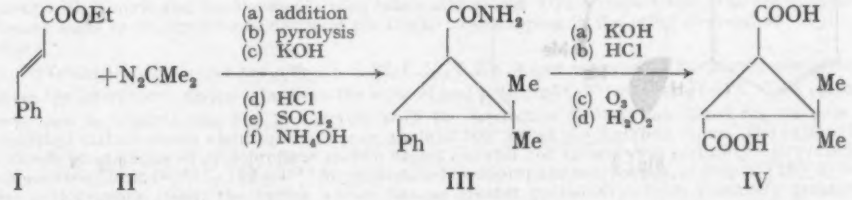
Die ontleding van die moontlike ruimtelike verdeling van die betrokke atoomgroeperings toon dat die siklopropaanring ook in hierdie geval eenvlakkig met die benseenring en die amied-karbonielgroep kan verkeer en so die deurkonjugeerde sisteem kan lewer wat deur die K-band in die ultraviolet absorpsie van die stof aangetoon word.

SUMMARY

The amide mentioned in the title was synthesized, starting from 2-diazopropane and ethyl cinnamate. Its structure was confirmed by degradation, *via* ozonolysis, to *trans*-caronic acid.

Consideration of the possible spatial distribution of the atomic groupings concerned shows that the cyclopropane ring can also in this case assume coplanarity with the benzene ring and the amide carbonyl group and so form the through-conjugated system indicated by the K band in the ultraviolet absorption of the amide.

A synthesis of *trans*-2-phenyl-3,3-dimethylcyclopropanecarboxamide, previously[1] attempted by employing the Buchner reaction[2] of ethyl diazoacetate with ω,ω-dimethylstyrene, led to low yields of two very similar amides, $C_{12}H_{15}ON$, m.p.s 131° and 128°. Unpublished degradative work on one of these products has subsequently shown that considerable rearrangement occurred in the course of this synthesis, so that an unambiguous synthesis of *trans*-2-phenyl-3,3-dimethylcyclopropanecarboxamide became necessary. This has now been achieved *via* the addition of the unstable 2-diazopropane (II) to ethyl cinnamate (I). As in previous cases [1, 3] the structure (III) of this amide, $C_{12}H_{15}ON$, m.p. 140°, was confirmed by hydrolysis and ozonolysis, followed by oxidation, when *trans*-caronic acid (IV) was readily obtained.



I II III IV

The ultraviolet absorption spectrum (Fig. 1) of *trans*-2-phenyl-3,3-dimethylcyclopropanecarboxamide (III) showed a normally developed K band at 218 mμ (log ϵ 4·01) beside the expected benzenoid absorption at 260 and 266 mμ (log ϵ 2·38 and 2·36 resp.). The corresponding acid and ethyl ester (see Experimental Section)

showed similar absorption. In terms of the discussion put forward previously[1], this meant that coplanarity could also be achieved in this case between the benzene ring, the cyclopropane ring and the amide carbonyl group.

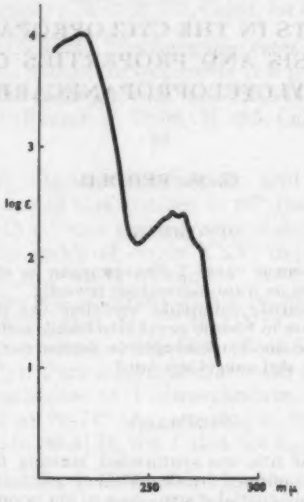


Fig. 1

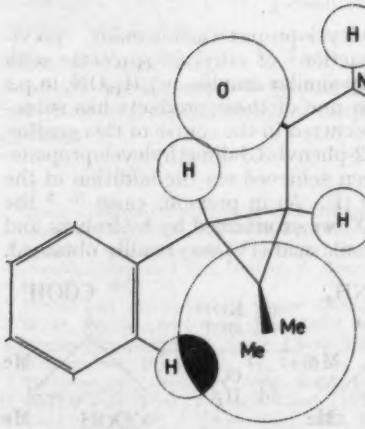


Fig. 2

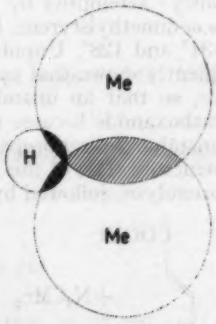


Fig. 3

The scale diagram of the plane molecule as previously given[4] indicated complete overlapping of the *ortho*-hydrogen atom of the phenyl group by the 3-*gem*-dimethyl groups, a condition which would prohibit coplanarity of the phenyl and cyclopropyl groups. It must, however, be pointed out that this planar diagram[4] represents an

extreme state of the spatial arrangement of the 3-*gem*-dimethyl groups. As the 3-carbon atom of the cyclopropane ring need have only two of its valency bonds in the plane containing the three unsaturated centres at coplanarity, the two remaining bonds of this atom may assume approximately tetrahedral distribution. The two 3-*gem*-dimethyl groups will then, in the most favourable configuration, occupy positions vertically above and below the plane of coplanarity and will, in projection on to this plane, appear closer to the 3-carbon atom than before.

This condition is represented in the scale diagram, Fig. 2*. While the projected 3-*gem*-dimethyl groups appear to overlap the *ortho*-hydrogen atom to a marked degree, this is in fact not so. An elevation† of this portion of the molecule, perpendicular to the axis of greatest crowding between these groups, shows (Fig. 3) that only slight overlapping occurs. Taking into account the considerably greater overlapping (Fig. 3, shaded area) between the 3-*gem*-dimethyl groups themselves, the repulsion between these two groups would lead to greater mutual separation than here shown, so that strain-free accommodation of the hydrogen atom in the coplanar arrangement of the molecule as a whole would clearly result.**

The light absorption properties of *trans*-2-phenyl-3,3-dimethylcyclopropanecarboxamide therefore agree with the hypothesis previously advanced for this series of compounds, *viz.* that the cyclopropane ring here functions as an unsaturated centre and that steric hindrance to the achievement of coplanarity between the three centres concerned fully explains the conjugation phenomena observed in all the members of this series so far studied.

EXPERIMENTAL

All m.p.s. are corrected. Ultraviolet absorptions were measured in ethanol solution using the Zeiss Opton Model M4Q spectrophotometer.

Dimethylketazine[6]. Acetone (62·8 g., 2 moles) was carefully added to 85 *per cent.* hydrazine hydrate (34·7 g.) with cooling. Water (38·5 ml.) was removed by azeotropic distillation under reflux after adding benzene (60 ml.) and the product (43·4 g.) distilled (Vigreux column) at 124-127°/655 mm. (Found: C, 64·4; H, 10·6. Calcd. for $C_6H_{12}N_2$: C, 64·2; H, 10·8%). *Acetone hydrazone* was prepared[7] from dimethylketazine (22 g.) and anhydrous[8] hydrazine (6·3 g.). Yield, 15·2 g., b.p. 117-119°/655 mm. The product was used without delay.

Ethyl 2-phenyl-3, 3-dimethylcyclopropanecarboxylate. Acetone hydrazone (15·0 g.) in dry toluene (50 ml.) was cooled to -10° to -15° and vacuum-dried yellow mercuric

*This diagram has been constructed using the dimensions previously given[1], except that the values for the covalent bond radii of the hydrogen atom and the methyl group have here, in agreement with Braude and Sondheimer[5], been taken as 0·60 and 1·70 Å respectively. The use of these values leads to no significant change in the relative overlapping in the other compounds[1] of this series.

†Triangulation around the *ortho*-H, C.2″, C.1″, C.2, C.3 and projected C.3‴ atomic positions gives the interatomic distance between the *ortho*-H and projected C.3‴ atoms as 1·67Å. This value was used in constructing Fig. 3, together with the separation (2·52Å) calculated for the *gem*-dimethyl carbon atoms when subtending an angle of 109° 28′ at the 3-carbon atom. The external valence bond angles of cyclopropane carbon atoms are still not known with certainty. Reported values range from 96°[12] to 112 ± 4°[13] for substituted cyclopropane compounds, and up to 118 ± 2°[14] for cyclopropane itself, the higher values having greater probability. Such relatively greater separation of the *gem*-dimethyl groups would enable the *ortho*-H atom to be accomodated even more favourably than here shown.

**Similar interrelationships hold for *trans*-2-(*o*-tolyl)-cyclopropanecarboxamide. Calculations carried out as above, however, show that here, in agreement with the experimental findings[1], considerable steric interference occurs even under the most favourable conditions.

oxide[9] (52·5 g.) added in small portions with vigorous evolution of gas. After 30 mins. at 10°, the initially colourless solution over the voluminous precipitate of metallic mercury and its oxide had turned reddish and acquired the pungent odour of the diazo compound. This solution of 2-*diazopropane*[10, 11] was cooled to below 0° and ethyl cinnamate (10 g.), b.p. 120·8-121·3°/5 mm., n_D^{20} 1·5594 (*lit.*, 1·5598), added, the mixture warming up spontaneously to about 45°. After standing at room temperature over-night, it was decanted through a filter bed of silica gel and washed with methanol. The filtrate was evaporated *in vacuo* to yield 15·5 g. syrupy residue which was heated from 160° to 270° (bath) over 80 mins., 1635 ml. gas (ambient conditions) being released without any appreciable distillation taking place. Two distillations *in vacuo* gave ethyl 2-phenyl-3, 3-dimethylcyclopropanecarboxylate (7·7 g.), fragrant liquid, b.p. 85-93° (bath)/0·001 mm., n_D^{20} 1·5091, $\lambda_{max.}$ 218 mμ (log ε 4·15) (Found: C, 77·0; H, 8·4. Calcd. for $C_{14}H_{18}O_2$: C, 77·0; H, 8·3%).

Trans-2-*phenyl*-3, 3-*dimethylcyclopropanecarboxamide*.The foregoing ester (5·07 g.) in ethanol (25 ml.) was refluxed for 17 hr. with potassium hydroxide (5·0 g.) in water (15 ml.). The solution was distilled with steam, the alkaline aqueous solution extracted with ether and then acidified with 10N hydrochloric acid (15 ml.). The acid product was collected in ether and obtained as a yellowish syrup (4·3 g.) which gradually crystallised.

The acid was all disolved in thionyl chloride (20 ml.), refluxed for 100 mins. and the excess reagent evaporated at 50°/30 mm. The crude acid chloride in acetone (5 ml.) was cautiously added to cooled concentrated ammonia liquor (35 ml.). The crude amide was collected in ether, dried (3·1 g.) and then sublimed for 13 hr. at 115°/0·005 mm. to give 2·64 g. sublimate. Crystallisation from aqueous ethanol and from benzene and ligroin gave 1·2 g. pure *trans*-2-phenyl-3, 3-dimethylcyclopropanecarboxamide, m.p. 140°, $\lambda_{max.}$ mμ/log ε: 218/4·01, 260/2·38, 266/2·36 (Found: C, 76·1; H, 7·9; N. 7·2. Calcd. for $C_{12}H_{15}ON$: C, 76·15; H, 8·0; N,7·4%).

Trans-2-*phenyl*-3, 3-*dimethylcyclopropanecarboxylic acid*. The foregoing amide (430 mg.) was refluxed with potassium hydroxide (2.0 g.) in water (20 ml.) for 20 hr. The clear solution was extracted with ether, acidified with 10N hydrochloric acid (15 ml.) and the acid recovered *via* ether extraction as 435 mg. colourless crystals, m.p. 103° unchanged on crystallisation from benzene and petroleum ether, $\lambda_{max.}$ mμ/log ε: 215/4·00 (broad), 259/2·35, 265/2·32, 272/2·03 (shoulder) (Found: C, 75·9; H, 7·4; neutr. eq. 189. Calcd. for $C_{12}H_{14}O_2$: C, 75·8; H, 7·4; neutr. eq., 190.)

Ozonolysis and oxidation of trans-2-*phenyl*-3, 3-*dimethylcyclopropanecarboxylic acid to* trans-*caronic acid*. Crude ozonized oxygen (2·4 vol. *per cent.* O_3, 0·2 l./min.) was bubbled through a solution of the above acid (153 mg.) in glacial acetic acid (20 ml.) for 4 hr. at 23°. The solution was then twice treated with 6 ml. 10 *per cent.* aqueous hydrogen peroxide, each time evaporating to dryness on a steam cone, to yield colourless crystals (107 mg.). Extraction with 2-butanone and sublimation up to 200°/0.05 mm. gave 85 mg. sublimate, which from 2-butanone and benzene and from water gave *trans*-caronic acid (47 mg.), colourless prisms, m.p. 217° alone and when mixed with the authentic compound (below) (Found: C, 53·2; H, 6·45; neutr. eq., 82. Calcd. for $C_7H_{10}O_4$: C, 53·2; H, 6·4; neutr. eq., 79).

Trans-caronic acid was obtained, for reference purposes, by reaction of 2-diazopropane with diethyl fumarate[11] as above, as colourless prisms, m.p. 217° (Found: C, 53·1; H, 6·6. Calcd. for $C_7H_{10}O_4$: C, 53·2; H, 6·4%).

Thanks are expressed to Miss J. M. Theron and Dr. A. P. Steyn for micro-analyses and spectral measurements respectively, as well as to student worker S. J. van der Walt (University of Pretoria) for preparative assistance in the synthesis of *trans*-caronic acid. Dr. T. J. W. Jorden is thanked for his interest and for permission to publish this paper.

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ULTRACENTRIFUGE STUDIES ON SEED PROTEINS OF THE FAMILY LEGUMINOSAE.

PART III. PROTEINS FROM DIFFERENT BEAN SPECIES

by

F. J. JOUBERT

OPSOMMING

Die eiwitte van die boontjie-varieteite van die geslag *Phaseolus vulgaris* was baie eenders. Elk bevat 'n hoofkomponent ($S_{6\cdot5}$) as vernaamste bestanddeel. 'n Ongewone assosiasie-dissosiasie-reaksie is waargeneem vir sommige varieteite van hierdie geslag. Die eiwitte van die kafferboontjie varieteite van die geslag *Vigna unguiculata* bevat 'n hoofkomponent ($S_{7\cdot3}$) wat omkeerbaar gedissosieer kan word. Die eiwitte van die geslag *Vicia faba* verskil van die ander boontjies daarin dat hulle twee hoofkomponente ($S_{11\cdot0}$ en $S_{7\cdot0}$) bevat, wat ontdaan van enige dissociasie-assosiasie eienskappe.

SUMMARY

The proteins from the bean varieties of the species *Phaseolus vulgaris* are very similar, each containing a major $S_{6\cdot5}$ component. An unusual association-dissociation reaction is found in the proteins of varieties of this species. The proteins from the cow pea varieties of the species *Vigna unguiculata* contain a major $S_{7\cdot3}$ component which can be reversibly dissociated. The proteins from the bean variety of the species *Vicia faba* contain two major $S_{11\cdot5}$ and $S_{7\cdot0}$ components which are devoid of any dissociation-association properties.

Investigation of the proteins of various beans has been carried out by Osborne[1] who, apart from elementary analyses, studied chiefly the solubility and coagulation properties of the globulins. In their chemical examination of the proteins of various beans, Johns and co-workers[2, 3, 4, 5, 6] have shown that nearly all of them contain two globulins, one occurring in relatively small amounts and the other constituting the chief protein of the seeds. Bourdillon[7] has succeeded in obtaining a crystalline protein from commercial dried beans (*Phaseolus vulgaris*), while Danielsson[8] has examined the proteins from single varieties of a number of bean species along with other seed globulins in the ultracentrifuge.

This paper deals mainly with an ultracentrifuge examination of the proteins from eight bean varieties of the species *Phaseolus vulgaris*. The results are compared with the sedimentation diagrams of the proteins from other bean species, and the effects of different ionic environments on the various protein components are reported.

EXPERIMENTAL

Sedimentation studies and dialysis of protein solutions were carried out as described previously[9].

Preparation of proteins. Table I gives the bean varieties examined and the protein content of the bean meals which were defatted by repeated extraction with hexane. Using a meal: liquor ratio of 1 : 10, the defatted bean meals were completely extracted with three separate volumes of 10 *per cent.* sodium chloride solution over a period of 24 hours at room temperature. The final protein extracts were centrifuged clear at 2,500 r.p.m. and the proteins precipitated with ammonium sulphate (85 *per cent.* saturation).

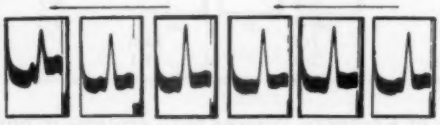
TABLE I

Protein contents of the different bean meals

Species	Variety	% Protein
Phaseolus vulgaris	Kidney	21·6
	Surecrop yellow podded	28·8
	Haricot	26·5
	Victory	24·3
	Speckled Sugar	24·4
	Black Wonder	25·4
	Lazy Housewife	24·9
	Kentucky Wonder	24·7
Phaseolus lunatus	Johnson's Lima	29·9
Vigna unguiculata (Cow pea)	Bechuana White	—
	Saunders' Upright	—
Vicia faba	Broad Bean	29·4

RESULTS AND DISCUSSION

Phaseolus Vulgaris, Phaseolus Lunatis *and* Vigna Unguiculata *Bean Species.* The proteins were dissolved and dialysed against various buffers. The sedimentation diagrams in buffer I=0·31, pH=7·0 are shown in Figs. 1 and 2. Sedimentation constants of the various components are listed in Tables II and III. For examination in different ionic environments, no attempt was made to separate the major $S_{6\cdot5}$ from the minor components since the former was the dominant component of all the bean varieties examined. As may be seen from Table III, no significant change in the sedimentation diagrams of the *Phaseolus vulgaris* and *Phaseolus lunatus* species occurred in buffer I=0·1, pH=9·0 as compared with those in buffer I=0·31, pH=7·0. The sedimentation diagrams in buffer I=0·11, pH=7·0, compared with those in buffer I=0·31, pH=7·0, were similar for most of the *Phaseolus vulgaris* and *Phaseolus lunatus* species, but in the case of the Haricot bean proteins a small concentration (10 *per cent.*) of a new component (17·6 S.U.) was observed. Appreciable quantities (20 *per cent.*) of this component were also found in Kentucky Wonder bean proteins in buffer I=0·11, pH=7·0.



(a) (b) (c) (d) (e) (f)

Fig. 1

Sedimentation diagrams of: (a) Kidney (b) Surecrop yellow podded (c) Haricot (d) Victory (e) Speckled Sugar and (f) Black Wonder bean proteins in buffer I=0·31, pH=7·0.

(a) (b) (c) (d) (e)

Fig. 2

Sedimentation diagrams of: (a) Lazy Housewife (b) Kentucky Wonder (c) Johnson's Lima bean proteins (d) Bechuana white and (e) Saunders' Upright cow pea proteins in buffer I=0·31, pH=7·0.

TABLE II

Sedimentation constants of the protein components of different beans in phosphate buffer I=0·31, pH=7·0.

Variety	Sedimentation constants (S°_{20} values)		
Kidney	(9·4),*	6·5,	(1·9)
Surecrop yellow podded	(9·1),	6·5,	(2·2)
Haricot	(9·3),	6·3,	(2·1)
Victory	(9·2),	6·5,	(2·0)
Speckled Sugar	(9·1),	6·6,	(1·9)
Black Wonder	(9·3),	6·6,	(2·2)
Lazy Housewife	(9·1),	6·5,	(2·1)
Kentucky Wonder	(9·4),	6·4,	(1·9)
Johnson's Lima	(9·2),	6·3,	(2·4)
Bechuana White	(9·3),	7·3,	(2·2)
Saunder's Upright	(9·1),	7·3,	(2·1)

*Sedimentation constants in brackets represent minor components.

TABLE III

Sedimentation constants of the major protein components of the different beans in various buffers.

Variety	Sedimentation constants (S°_{20} values)		
	I=0·31, pH=7·0	I=0·1, pH=9·0	I=0·11, pH=7·0
Kidney	6·5	6·8	7·0
Surecrop yellow podded	6·5	6·8	6·9
Haricot	6·3	6·7	17.8, 6·8
Victory	6·5	6·8	6·9
Speckled Sugar	6·6	6·8	7·0
Black Wonder	6·6	6·8	6·9
Lazy Housewife	6·5	6·8	6·9
Kentucky Wonder	6·4	6·7	17·6 6·8
Johnson's Lima	6·3	6·5	6·8
Bechuana White	7·3	11·0	11·3
Saunders' Upright	7·3	11·1	11·5

When a solution of Kentucky Wonder bean proteins in buffer I=0·31, pH=7·0 was dialysed overnight against buffer I=0·05, pH=7·0 [Fig. 3(b)], a minute amount of the $S_{17\cdot6}$ component formed. When dialysed against buffer I=0·1, pH=6·2, however, about 70 *per cent.* of the $S_{6\cdot5}$ component was converted into the $S_{17\cdot6}$ component [Fig. 3(c)]. When the latter solution was dialysed back to I=0·31, pH=7·0, the $S_{6\cdot5}$ component formed again (Fig. 3(d); compare with Fig. 3(a) which shows the original $S_{6\cdot5}$ component. The conversion of the $S_{6\cdot5}$ component into the $S_{17\cdot6}$ component was therefore reversible. These results are summarised in Table IV. Assuming the same value (1·2) for the frictional ratio for each sedimenting component, the sedimentation constants 6·5 and 17·6 S.U. correspond to molecular weights of *ca.* 116,000 and 482,000 respectively. Apparently four molecules of the lower molecular weight are thus involved in the association reaction.

In the *Vigna unguiculata* species, a marked change occurred when the buffer was changed from I=0·31, pH=7·0 to either I=0·1, pH=9·0 or I=0·11, pH=7·0. The major $S_{7\cdot3}$ component disappeared nearly completely and was replaced re-

spectively by $S_{11\cdot0}$ and $S_{11\cdot4}$ components. The change was completely reversible. The sedimentation constants of 7·3 and 11·4 S.U. correspond respectively to molecular weights of *ca.* 129,000 and 251,000 (assuming the same value of 1·2 for the frictional ratio for both components) and the $S_{7\cdot3}$ component therefore seemingly dimerised as the buffer was changed.

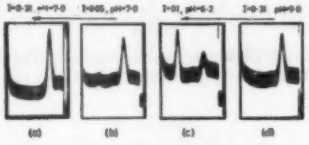


Fig. 3

Sedimentation diagrams of Kentucky Wonder bean proteins in different buffers.

TABLE IV

Sedimentation constants of the major protein components of Kentucky Wonder bean and Bechuana white cow pea in various buffers.

Variety		I	pH	Sedimentation constants (S°_{20} values)	
Kentucky Wonder		0·31	7·0		6·4
		0·05	7·0		7·1
		0·1	6·2	17·6,	6·8
	Dialysed back to:	0·31	7·0		6·8
Bechuana White		0·31	7·0		7·3
		0·1	9·0	11·1	
	Dialysed back to:	0·31	7·0		7·7

Fig. 4

Sedimentation diagrams of Bechuana white cow pea proteins in different buffers.

Vicia faba *species* (*Broad Bean*). Fig. 5 contains the sedimentation diagrams in phosphate buffer I=0·31, pH=7·0 of the protein which was precipitated from a 10 *per cent.* sodium chloride extract of Broad Bean meal by saturation to 85 *per cent.* with ammonium sulphate. At least four sedimenting components were found—two major components (11·5 and 7·0 S.U.) and two minor components (16·2 and 1·9 S.U.). Sedimentation diagrams of the various fractions (Table V), obtained by adding saturated ammonium sulphate to a 10 *per cent.* sodium Broad Bean protein extract, are shown in Fig. 5. The $S_{11\cdot5}$ and $S_{7\cdot6}$ components were obtained in a reasonable state of purity in the A_4 and A_6 fractions respectively. The latter fractions showed appreciable change in buffers I=0·31, pH=7·0; I=0·1, pH=9·0 and I=0·11, pH=7·0 (Table VI).

The proteins from the *Vicia faba* bean species differed from the other beans in that they contained two major sedimenting components. These two components were devoid of any dissociation-association reactions within the range of experimental conditions.

Fig. 5

Sedimentation diagrams of ammonium sulphate fractions of Broad bean proteins in phosphate buffer I=0·31, pH=7·0.

TABLE V

Ammonium sulphate fractionation of Broad Bean proteins

Fraction	% Saturation with ammonium sulphate	% of total precipitated protein	Sedimentation constants (S°_{20} values)			
Total	—	—	·(16·2),	11·5,	7·0,	(1·9)
A_1	40	9	—	—	(6·9),	(3·6)
A_2	50	3	— ,	(11·7),	6·8,	(2·2)
A_3	55	46	(16·0),	11·8,	(7·0),	(2·1)
A_4	60	22	— ,	11·8,	(7·1),	—
A_5	70	2	— ,	(12·1),	7·2,	(1·9)
A_6	85	18	— ,	— ,	6·8,	(2·0)

TABLE VI

Sedimentation constants of Broad Bean protein fractions in different buffers.

Fraction	Sedimentation constants (S°_{20} values)					
	I=0·31, pH=7·0		I=0·1, pH=9·0		I=0·11, pH=7·0	
A_4	11·8		12·3		12·8	
A_6		6·8		7·6		7·5

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ULTRACENTRIFUGE STUDIES ON SEED PROTEINS OF THE FAMILY LEGUMINOSAE. PART IV. THE ISOLATION AND PHYSICO-CHEMICAL EXAMINATION OF THE GLOBULINS FROM WHITE LUPIN SEED

by

F. J. JOUBERT

OPSOMMING

In die ultrasentrifuge toon die globuliene van wit lupiensaad een hoofkomponent ($S_{8\cdot3}$) en kleiner hoeveelhede van $S_{12\cdot1}$- en $S_{2\cdot8}$-komponente. Redelik suiwer $S_{8\cdot3}$- en $S_{2\cdot8}$-Komponente is verkry deur gebruik te maak van ammoniumsulfaatfraksionasie. Die $S_{12\cdot1}$ an $S_{3\cdot8}$ komponente is geskei deur die iso-elektriese pH presipitasie-metode. Die $S_{12\cdot1}$- en $S_{8\cdot3}$-komponente vorm beide omkeerbare dissosiasie-assosiasie-systeme. Die dissosiasie-assosiasie-reaksie van die $S_{12\cdot1}$-komponent verskil van dié van die $S_{8\cdot3}$-komponent; die $S_{12\cdot1}$-komponent dissosieer as die buffer verander word van I=0·31, pH=7·0 na I=0·1, pH=9·0, terwyl die $S_{8\cdot3}$- komponent assosieer. Die dissosiasie-assosiasiereaksie van die $S_{12\cdot1}$-komponent word in die eerste plek beïnvloed deur pH in dié van die $S_{8\cdot3}$-komponent deur ioniese sterkte. Die globuliene van wit lupiensaad is vergelyk met dié van blou en geel lupiensaad wat alreeds beskrywe is.

SUMMARY

In the ultracentrifuge the globulins from white lupin seed show one major ($S_{8\cdot3}$) component and two minor ($S_{12\cdot1}$ and $S_{2\cdot8}$) components. Reasonably pure $S_{8\cdot3}$ and $S_{2\cdot8}$ components are obtained by ammonium sulphate fractionation. The $S_{12\cdot1}$ and $S_{8\cdot3}$ components are separated by the isoelectric pH precipitation method. The latter components both form reversible dissociation-association systems, but whereas the $S_{12\cdot1}$ component dissociates in going from I=0·31, pH=7·0 to I=0·1, pH=9·0, the $S_{8\cdot5}$ component associates. The dissociation-association reaction of the $S_{12\cdot1}$ component is primarily influenced by pH and that of the $S_{8\cdot3}$ component by ionic strength. The globulins of white lupin seed are compared with those of blue and yellow lupins previously reported.

While the proteins of two other lupins, *viz.*, *Lupinus angustifolius*[1] and *Lupinus luteus*[2, 3] have been studied in detail by ultracentrifugal methods, little work has been done on the proteins of the white lupin (*Lupinus albas*). Danielsson[4] has examined the unfractionated proteins from the white lupin along with other seed globulins of the family Leguminosae in the ultracentrifuge and reported sedimentation data for this lupin.

This paper is mainly concerned with the fractionation of the proteins in white lupin seed meal and the ultracentrifugal study of the effect of various ionic environments on these fractions. In addition to sedimentation studies, measurements have also been made by diffusion and electrophoresis. Preliminary results on white lupin seed globulins have already been published[5].

EXPERIMENTAL

Ultracentrifuge, electrophoretic and diffusion measurements were made with the apparatus described previously[1, 3], the electrophoretic experiments all being carried out at 20°.

Preparation of Proteins. Decorticated white lupin seed meal was defatted and purified as previously described[1, 3]. The proteins from the defatted meal and the

purified defatted meal were extracted with 10 *per cent.* sodium chloride and precipitated by adding ammonium sulphate to 85 *per cent.* saturation [1, 3].

RESULTS AND DISCUSSION

Fig. 1(a) and 1(b) are sedimentation diagrams of these two preparations (see Experimental) dialysed against phosphate buffer I=0·31, pH=7·0. In both cases three sedimenting components, one major (sedimentation constant 8·3 Svedberg Units) and two minor components (12·1 and 2·8 S.U.), were noticed, a slight decrease in the concentration of the $S_{2\cdot8}$ component occurring in the purified meal protein (compare Figs. 1(a) and 1(b). The proteins extracted by the wash water during purification of the meal were precipitated at 85 *per cent.* saturation with ammonium sulphate and, as shown in Fig. 1(c), consisted mainly of the slowly sedimenting material.

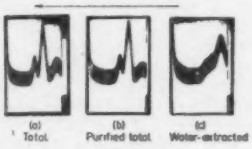

Fig. 1

Sedimentation diagrams of white lupin seed proteins in phosphate buffer I=0·31, pH=7·0.

The proteins of the defatted meal and purified defatted meal were also examined electrophoretically in borate buffer I=0·1, pH=9·0. The preparations gave similar electrophoretic diagrams [Fig. 2(a) and 2(b)] and the electrophoretic mobilities (Table I) of the different components were comparable. The purification of the meal (removal of colouring matter) therefore had no appreciable effect on the proteins. The electrophoretic diagrams (Fig. 2(a) and 2(b)) show distinctly three components and a small concentration of a slow component. The components of higher electrophoretic mobility seem to agree with the three sedimenting components observed in the ultracentrifuge [Fig. 1(a) and 1(b)]. Furthermore, a small amount of an $S_{10\cdot7}$ component was isolated from white lupin seed globulins by alcohol fractionation. This probably accounts for the low concentration of the electrophoretically slow component which would not show up in the sedimentation diagrams (Fig. 1(a) and 1(b)).

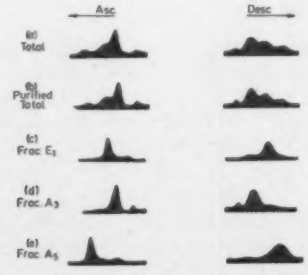

Fig. 2

Electrophoretic diagrams of white lupin seed proteins in borate buffer I=0·1, pH=9·0.

Fractionation of White Lupin Seed Globulins. A preliminary fractionation of white lupin seed globulins was achieved by the step-wise addition of saturated ammonium sulphate to a 10 *per cent.* sodium chloride extract of the purified white lupin seed (Table II) (sedimentation diagrams are shown in Fig. 3). This fractionation showed the $S_{8\cdot3}$ component to be the major component of the globulins; only a relatively small amount of the pure $S_{8\cdot3}$ component could, however, be isolated, while the $S_{2\cdot8}$ component was fairly pure in Fraction A_5.

TABLE I

Electrophoretic mobilities of white lupin seed proteins at 20°C, in borate buffer I=0·1, pH=9·0.

Preparation	Mobility x 10^{-4} cm^2 sec^{-1} $volt^{-1}$							
	Descending				Ascending			
	$S_{10\cdot7}$	$S_{8\cdot3}$	$S_{12\cdot2}$	$S_{8\cdot2}$	$S_{10\cdot7}$	$S_{8\cdot3}$	$S_{12\cdot2}$	$S_{8\cdot2}$
Total	(0·24),*	0·62,	0·98,	(1·45)	(0·33),	0·80,	1·20,	(1·57)
Purified Total	(0·24),	0·64,	0·96,	(1·40)	(0·32),	0·78,	1·15	(1·56)
Fraction E_1			1·08				1·21	
Fraction A_3		0·66				0·70		
Fraction A_5				1·47				1·70

*Mobilities in brackets represent minor components.

TABLE II

Ammonium sulphate fractionation of white lupin seed globulins dissolved in 10 *per cent.* sodium chloride

Fraction	% Saturation with ammonium sulphate	% of total precipitated protein	Sedimentation constants (S°_{20} values)		
A_1	31	5	12·2,	(8·4)*	
A_2	40	65	(12·4),	9·0	
A_3	50	15		8·6	
A_4	70	5		8·8,	(2·8)
A_5	85	10		(8·6),	2·8

*Sedimentation constants in brackets represent minor components.

When a 10 *per cent.* sodium chloride extract of the purified white lupin seed meal was saturated to 50 *per cent.* with ammonium sulphate, 85 *per cent.* of the extracted proteins were precipitated (Fraction I). Fraction I contained only the $S_{12\cdot1}$ and $S_{8\cdot3}$ components. The protein precipitated from a 10 *per cent.* sodium chloride solution of this fraction by ammonium sulphate was examined ultracentrifugally, the results being shown in Table III. Similarly, no significant separation of the $S_{12\cdot1}$ and $S_{8\cdot3}$ components could be achieved by low temperature alcohol fractionation, the results of which are shown in Tables IV and V.

The most effective separation was achieved by a method based on the precipitation of the heavier component at a pH in the vicinity of its iso-electric point. Fraction I was dissolved in 5 *per cent,* 10 *per cent* and 15 *per cent.* sodium chloride solutions and each adjusted to pH 4·0 with acetate buffer (I=0·1, pH=4·0). The precipitates which formed were centrifuged off and the supernatants saturated to 85 *per cent.* with ammonium sulphate to precipitate a second protein fraction. The amounts and sedimentation constants of each fraction are given in Table VI. The $S_{12}{}'_1$ component was reasonably pure in Fraction E_1 and Fractions E_4 and E_6 contained only the $S_8{}'_3$ component.

Thus by using both ammonium sulphate fractionation and the iso-electric pH precipitation method, the $S_{12\cdot1}$, $S_{8\cdot3}$ and $S_{2\cdot8}$ components were obtained in a reasonable state of purity. The three fractions containing the $S_{12\cdot1}$, $S_{8\cdot3}$ and $S_{2\cdot8}$ components (E_1, A_3 and A_5 respectively) were subjected to a further examination. Fig. 2(c), 2(d) and 2(e) show respectively electrophoretic diagrams of Fractions E_1, A_3 and A_5 and the electrophoretic mobilities of the various fractions are given in Table I. These fractions were also reasonably pure as shown by electrophoresis.

Fig. 3

Sedimentation diagrams of Ammonium sulphate fractions of white lupin seed globulins in phosphate buffer I=0·31, pH=7·0.

TABLE III

Ammonium sulphate fractionation of Fraction I dissolved in 10 *per cent.* sodium chloride.

Fraction	% Saturation with ammonium sulphate	% of total precipitated protein	Sedimentation constants* (S°_{20} values)	% of Fast Comp.	% of Slow Comp.
B_1	27		12·5 , (8·5)	66	34
B_2	30·2	1	12·6 , (8·6)	60	40
B_3	31·7	6	12·4 , 8·4	46	54
B_4	33·3	19	12·2 , 8·3	42	58
B_5	34·9	23	12·0 , 8·2	37	63
B_6	36·5	13	(12·2), 8·2	22	78
B_7	38·7	12	(12·3), 8·1	14	86
B_8	42·6	20	(12·0), 8·0	6	94
B_9	50	5	— , 7·9	0	100

TABLE IV

Low temperature alcohol fractionation of Fraction I dissolved in 10 *per cent.* sodium chloride

Fraction	% Alcohol added	% of total precipitated protein	Sedimentation constants* (S°_{20} values)	% of Fast Comp.	% of Slow Comp.
C_1	6·0	1	(12·6), 8·4	34	66
C_2	6·9	1	(12·7), 8·3	42	58
C_3	8·7	36	(12·5), 8·4	26	74
C_4	10·3	29	(12·4), 8·3	24	76
C_5	11·8	16	(12·3), 8·4	27	73
C_6	13·2	6	(12·2), 8·3	29	71
C_7	15·0	5	(12·0), 8·1	26	75
C_8	19·3	4	(12·0), 8·0	14	86
C_9**	41·0	2	(10·8),‡ 7·9	31	69

*In phosphate buffer, I=0·31, pH=7·0.
**2 *per cent.* of total precipitated protein.
‡Probably a minor component.

Examination of Fractions E_1 and A_3 in different buffers. The ionic strength and pH of a solution of Fraction E_1 was varied by dialysis (See Table VII). Fig. 4(a), showing a major $S_{12\cdot1}$ component, gives the sedimentation diagram of Fraction E_1 in phosphate buffer I=0·31, pH=7·0. This solution was dialysed for 16 hours against

borate buffer I=0·1, pH=9·0 [Fig. 4(b)] when about 70 *per cent.* of the $S_{12\cdot1}$ component disappeared and was replaced by a component of sedimentation constant 7·5 S.U. Further storage [44 hours, Fig. 4(c)] at room temperature led to no apparent change The latter solution was dialysed back to I=0·31, pH=7·0 and, as shown in Fig. 4(d) the $S_{12\cdot1}$ component formed again, the sedimentation diagrams being identical with the original diagrams [compare Fig. 4(a) and 4(d)]. Hence Fraction E_1 formed a reversible dissociation-association system.

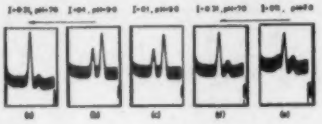

Fig. 4

Sedimentation diagrams of Fraction E_1 in different buffers.

TABLE V

Low temperature alcohol fractionation of Fraction I dissolved in phosphate buffer I=0·31, pH=7·0

Fraction	% Alcohol added	% of total precipitated Protein	Sedimentation constants (S°_{20} values)	% of Fast Comp.	Slow Comp.
D_1	0	28	(12·3), 8·7	26	74
D_2	1·0	19	(12·1), 8·5	21	79
D_3	2·2	14	(12·4), 8·6	18	82
D_4	3·6	12	(12·2), 8·6	20	80
D_5	5·6	14	(12·1), 8·3	31	69
D_6	10·2	13	11·9, (10·6), 7·8	43 21	36

TABLE VI

Iso-electric pH precipitation of Fraction I dissolved in different sodium chloride solutions.

Fractions	Sodium chloride concentration	% of total precipitated protein	Sedimentation constants (S°_{20} values)	% if Fast comp.	Slow comp.
E_1 pH=4·0 ppt.	5%	15	12·1 , (8·0)	94	6
E_2 Supernatant	5%	85	(12·1), 8·0	9	91
E_3 pH=4·0 ppt.	10%	27	12·2 , (8·5)	61	39
E_4 Supernatant	10%	73	— , 8·1	0	100
E_5 pH=4·0 ppt.	15%	53	11·9 , 8·2	44	56
E_6 Supernatant	15%	47	— , 8·0	0	100

Fraction E_1 was subsequently examined in phosphate buffer I=0·11, pH=7·0, i.e. at the same pH but lower ionic strength. The sedimentation diagrams obtained [Fig. 4(e)] show a major $S_{12\cdot1}$ component and were similar to the diagrams at I=0·31, pH=7·0. A small concentration of a component of sedimentation constant slightly higher than 12·1 S.U. may be seen in the sedimentation diagrams of Fig. 4(e), but the significance of this is not at present known. At constant ionic strength of $\pm$0·1 and on increasing the pH from 7·0 to 9·0, dissociation of Fraction E_1 occurred. Thus, within the range of experimental conditions, association-dissociation was primarily influenced by pH and very little, if at all, by ionic strength.

Similarly Fraction A_3, containing a single $S_{8\cdot3}$ component [Fig. 5(a)] in phosphate buffer I=0·31, pH=7·0, was dialysed overnight against borate buffer I=0·1, pH=

9·0 [Fig. 5(b)] and thereby almost quantitatively converted into a component of 12·5 S.U. (see Table VII). When this solution was dialysed back to I=0·31, pH=7·0, a single $S_{8\cdot3}$ component formed again as shown in Fig. 5(c). The sedimentation diagrams obtained were identical with the original diagrams [compare Fig. 5(a) and 5(c)]. Hence the protein in Fraction A_3 also formed a reversible association-dissociation system, but in contrast to E_1 it associated in going from I=0·31, pH=7·0 to I=0·1, pH=9·0, whereas E_1 dissociated.

Fig. 5

Sedimentation diagrams of Fraction A_3 in different buffers.

TABLE VII

Sedimentation constants of major components of Fractions E_1 and A_3 in different buffers.

Fraction		I	pH	Sedimentation constants (S°_{20} values)	
E_1		0·31	7·0	11·8	
E_1		0·1	9·0	11·7	7·4
E_1	Dialysed back to	0·31	7·0	12.0	
E_1		0·11	7·0	12·4	
A_3		0·31	7·0		8·3
A_3		0·1	9·0	12·2	
A_3	Dialysed back to	0·31	7·0		8·3
A_3		0·11	7·0	12·5	
A_3	Dialysed back to	0·31	7·0		8·4

TABLE VIII

Sedimentation constants of Fractions E_1 and A_3 at different concentrations in various buffers.

Fraction E_1		Fractions A_3			
I=0·31,	pH=7·0	I=0·31,	pH=7·0	I=0·11,	pH=7·0
Conc. %	S°_{20}-values	Conc. %	S°_{20}-values	Conc. %	S°_{20} values
1·25	11·8	1·30	8·5	1·31	12·6
1·00	12·0	0·92	8·2	0·92	12·5
0·62	12·2	0·63	8·4	0·60	12·4
0·31	12·5	0·30	8·1	0·30	12·4

Fraction A_3 was further examined in phosphate buffer I=0·11, pH=7·0 when a major $S_{12\cdot8}$ component appeared [see Fig. 5(d)]. On dialysing back to I=0·31, pH=7·0, Fig. 5(c) was reproduced. The association-dissociation reaction shown by Fraction A_3 was therefore influenced primarily by ionic strength.

Molecular weights of fractions E_1 and A_3. Table VIII contains sedimentation constants (S°_{20} values) of Fraction E_1 in buffer I=0·31, pH=7·0 and of Fraction A_3 in buffers I=0·31, pH=7·0 and I=0·11, pH=7·0. No appreciable change in the sedimentation constants of Fraction A_3 with concentration was found. The sedimentation

constants of Fraction E_1, however, increased with decreasing concentration. When these results were extrapolated to zero concentration, a value of 12·6 S.U. was found for the sedimentation constant of Fraction E_1.

Diffusion coefficients were determined at 20° for Fraction E_1 in buffer I=0·31, pH=7·0 and for Fraction A_3 in buffers I=0·31, pH=7·0 and I=0·11, pH=7·0. The results were calculated by the standard deviation method and are shown in Table IX. The molecular weights and frictional ratios of Table IX were calculated from the diffusion coefficients and the sedimentation constants in Table VIII. The molecular weight of assocated Fraction A_3 in buffer of I=0·11, pH=7·0 was somewhat lower than that of Fraction E_1 in buffer of I=0·31, pH=7·0 and very much higher than that of Fraction A_3 in buffer of I=0·31, pH=7·0, although not exactly double.

TABLE IX

Molecular weights of Fractions E_1 and A_3 in various buffers

	Fraction E_1	Fraction A_3	
	I=0·31, pH=7·0	I=0·31, pH=7·0	I=0·11, pH=7·0
$D^{\circ}_{20}cm^2sec^{-1}$	$2{\cdot}99 \times 10^{-7}$	$3{\cdot}80 \times 10^{-7}$	$3{\cdot}39 \times 10^{-7}$
S°_{20} Svedberg Units	12·6	8·3	12·5
Partial specific volume (assumed)	0·74	0·74	0·74
Molecular weight	393,000	204,000	344,009
Frictional ratio	1·46	1·43	1·35

TABLE X

Comparison of the sedimentation and electrophoretic properties of the globulins from the three different lupins.

Lupin	Component	Sedimentation Constants (S°_{20} values) in different buffers		Electrophoretic mobility x $10^{-4}cm^2sec^{-1}volt^{-1}$ in buffer I=0·1, pH=9·0	
		I=0·31, pH=7·0	I=0·1, pH=9·0	Descending	Ascending
Lupinus angustifolius (Blue)	1st Globulin	11·6	7·2*	1·16	1·33
	2nd Globulin	7·8	7·8	0·67	0·76
Lupius luteus (Yellow)	1st Globulin	11·6	7·2*	1·14	1·25
	2nd Globulin	7·4	7·9	0·76	0·86
	3rd Globulin	2·0	—	1·39	1·57
Lupinus albus (White)	1st Globulin	12·1	7·5*	1·08	1·21
	2nd Globulin	8·3	12·5*	0·66	7·70
	3rd Globulin	2·8	3·2	1·47	1·70

* Reversible dissociation-association systems.

Comparison of the physico-chemical properties of the globulins from the three lupins. In Table X sedimentation and electrophoretic constants of the globulins from the three lupins studied are summarised. In the case of the blue and yellow lupins, there are several points of similarity. Each contains a high molecular weight glubulin (first globulin) as main constituent which can be reversibly dissociated. From their physico-chemical properties, it would thus appear that the first globulins of blue and yellow lupins are identical. Gerritsen[6], however, found that the nitrogen contents of the two

globulins were different and also noticed differences in amino acid composition, the most striking being the number of arginine residues per molecule (222 for yellow lupin and 278 for blue lupin).

The sedimentation constants and electrophoretic mobilities of the second globulins of blue and yellow lupin were somewhat different. Furthermore, these globulins contained different amounts of most of the amino acids present in them (Gerritsen [6]). Although the sedimentation constants of the second globulins were similar to those of the dissociated parts of the first globulin, the second globulin was devoid of any association-dissociation properties. These conclusions were confirmed by Gerritsen [6], who observed that the amino acid composition of the dissociated parts of the first globulins of blue and yellow lupins was not identical with those of the second globulins. The most significant difference was that the first globulins contained tryptophan, which was completely absent in the second globulins.

On account of its association-dissociation behaviour, Fraction E_1 may be regarded as the first globulin of white lupin. It is, however, only a minor constituent and has somewhat higher sedimentation constants than those of blue and yellow lupins although its electrophoretic mobilities are similar. The second globulin in white lupin is represented by the protein in Fraction A_3. It differs from the second globulins of blue and yellow lupins in being the major constituent and in being an associating protein, though with association-dissociation properties different from the first globulins. The electrophoretic mobility of the second globulin resembles that of the second globulin of blue and yellow lupins, even though this was measured under conditions where it associated. The low molecular weight third globulin of white lupin is probably analogous to the third globulin of yellow lupin.

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STUDIES IN THE METABOLISM OF FUNGI. PART I. A CELLULOLYTIC ENZYME PREPARATION FROM *HYDNUM HENNINGSII* (Bres.)

by

T. A. KILROE-SMITH

OPSOMMING

'n Voorlopige ondersoek is na die voorkoms van die meer gewone ensiemstelsels in die twee houtvernietigende swamme, *P. rugulosis* Lev. en *H. henningsii* Bres. ingestel. 'n Gekonsentreerde sellulolitiese ensiemoplossing is uit laasgenoemde berei. Enkele eienskappe van hierdie ensiemoplossing is ondersoek. Die ensiem word vinnig onaktief by temperature bokant 50°, en in 'n alkaliese oplossing met pH bokant 6. Met hergepresipiteerde sellulose is die optimum toestande as volg: pH omstreeks 3, temperatuur omstreeks 37°. By betreklike lae ensiemkonsentrasies bestaan daar 'n byna volmaakte eerstemagsverhouding tussen ensiemkonsentrasie en aktiviteit. Die hoofproduk van die hidroliese van sellulose is glukose, maar 'n klein hoeveelheid sellobiose word ook gevind.

SUMMARY

A preliminary survey is made of the occurrence of some common enzyme systems in the two wood-destroying fungi *P. rugulosus* Lev. and *H. henningsii* Bres., and a cellulolytic enzyme concentrate is prepared from the latter. Some of the properties of this enzyme concentrate are investigated. The enzyme is rapidly inactivated at temperatures above 50° and in alkaline solution above pH 6. On reprecipitated cellulose the optimum conditions are at a pH of *ca.* 3 and *ca.* 37°. An essentially linear relationship exists between enzyme concentration and activity at relatively low enzyme concentration. The main hydrolysis product of cellulose is glucose, but a small amount of cellobiose is also found.

Two of the most prominent wood-destroying fungi found in the Witwatersrand gold mines are *Polyporus rugulosus* Lev., and *Hidnum henningsii Ares.* These two fungi belong to the two groups designated "white-rot" or lignin-destroying fungi, and "brown-rot" or cellulose-destroying fungi, respectively. To investigate the metabolism of these fungi a preliminary survey was necessary to ascertain the presence of a number of selected enzyme systems in these two fungi. While the substrate on which the fungus is grown can influence the development of the enzymes, a general idea of the type of enzymes present in the mycelium can nevertheless be obtained if the fungi are grown on a medium on which apparently normal growth occurs, namely, 2·0 *per cent.* malt agar. Nevertheless it is necessary to guard against generalisations when an enzyme system is not found in the mycelium grown on a specific medium, since it is possible that the system in question could be produced on a different medium.

Table I records the results of this preliminary survey. Although the list is by no means exhaustive, it nevertheless includes the most commonly occurring enzyme systems.

TABLE I

Enzymes in two wood-destroying fungi

Enzyme	*Polyporus Rugulosus* Lev. T.R.L. 25		*Hydnum Henningsii* Bres. T.R.L. 24		Refs.
	Extra-cellular	Intra-** cellular	Extra-cellular	Intra-** cellular	
Invertase	+		+(?)		15
Amylase	+		?*	?*	15
Cellulase	+		+		15
α-Methylglucosidase	+(?)		+		15
Maltase	−		+		15
Lactase	−	−	?		15
Esterase	−	−	−		15
Lipase	−	−	−		15
Urease	−	?	−*	−*	5
Catalase	+		−*	−*	15
Phenoloxidase	+		−*	−*	†
Alcohol oxidase	−	−	−	+	‡
Peroxidase	−	+	−	+	5
Dehydrogenase	−	+	−	+	5

* Oxalic acid and/or dark colour and/or methods of removal of same may have masked these tests.
** Done only if the extracellular test was negative.
† Detected by darkening of colour when the enzyme solution was incubated with a 1 *per cent.* phenol solution at 30° for a few hours.
‡ Detected by distilling off any acetaldehyde formed after incubation at 30° with 3 *per cent.* ethanol. Aldehyde detected with hydroquinone[4].

In the attack on wood by a cellulose-destroyer the first enzyme in the metabolic series would probably be a cellulase. Cellulase preparations have been obtained from a number of sources, including fungi, bacteria, animal life, higher plants and protozoa. Summaries of references are given by Saunders, Siu and Genest[8] and by Siu[11]. The enzyme responsible for cellulase activity, however, is not necessarily the same in each of these preparations, as is evident from the data summarised by Pigman[5] and by Siu[12] regarding pH and temperature effects. It was therefore desirable to examine the properties of the cellulase from *H. henningsii*.

EXPERIMENTAL

Preparation of Enzyme Solutions. The fungi selected for this investigation were taken from the culture collection of the Transvaal and Orange Free State Chamber of Mines Timber Research Laboratory and were designated T.R.L.24 (*Hydnum henningsii* Bres.) and T.R.L.25 (*Polyporus rugulosus* Lev.).

For the preliminary survey of enzymes, each fungus was allowed to grow on 2·0 *per cent.* malt agar until the surface of the agar was completely covered. The mycelium and malt agar were broken up and partially dried by suction. The residue (A) and liquid extract (B) were then separately dried in a vacuum over calcium chloride. Portions of dry solid from (A) and (B) were extracted separately with a minimum quantity of water and the extracts used for tests for extracellular exoenzymes.

Intracellular-endoenzyme solutions were prepared by grinding (A) with fine sand to a fine powder (100-mesh) and extracting with the minimum of 1·0 *per cent.* chloroform. Tests for intracellular enzymes were only done if negative results were obtained in the extracellular enzyme tests.

Owing to the dark colour of the solutions from *H. henningsii* it was not possible to do directly any of the tests depending on a change of colour. Hence, the solution was partly decolourised for these tests by stirring intermittently for 24 hours with animal charcoal. Tests were done on the filtered solution in the cases of urease, dehydrogenase and phenoloxidase. Some enzymes may have been adsorbed by the charcoal. Furthermore, owing to the formation of oxalic acid by *H. henningsii* on malt agar, it was not possible directly to detect peroxidase or catalase. For these two tests, therefore, the oxalic acid was removed as the calcium salt.

For the preparation of a cellulolytic enzyme concentrate from *H. henningsii* (T.R.L.24), cultures were grown at 27° and 85 *per cent.* relative humidity for nine months on *Eucalyptus saligna* sapwood shredded to a fine matchwood. At the end of this time considerable growth had taken place with the formation of sporophores in the medium in many cases. The mycelium on the surface of the wood, sporophores and the bulk of the medium were harvested separately and tested for cellulase activity. Since in some cases the woody medium was more heavily attacked than in others, a comparison was made between the heavily and the lightly attacked wood. Samples of each were extracted with 1 *per cent.* chloroform (18 hours at room temperature) and the derived liquid tested for cellulase activity by the standard method at pH 5·6 (see assay method below). Only a small amount of enzyme was found in the sporophores, the mycelium being at least five times as active. As expected, the more heavily attacked wood showed greater enzyme activity than the lightly attacked wood.

Cellulase assay. Enzyme activity was assayed by the increase in the Shaffer and Hartmann[10] reducing sugar titre given by the enzyme after 72 hours at 35° in a 1 *per cent.* dispersion of precipitated cellulose in a buffer solution, as compared with a control boiled for 10 minutes to destroy enzyme activity. Toluene was added as an antiseptic.

The precipitated cellulose was prepared by the method of Scales[9] from Whatman No. 1 filter paper. In the earlier experiments in this series the buffer used was a 0·05M acetate buffer at pH 5·6, following Whitaker[14]. Later, when it was found that the enzyme showed greater activity at lower pH, a 0·05M citrate buffer, the pH of which was adjusted to 3·2 with concentrated disodium phosphate, was used.

Properties of the enzyme concentrate. After adjusting the pH (originally ±3·0) to 5·5, the combined extracts were concentrated to about one twelfth of the original volume in a Bartholomew evaporator below 45°.

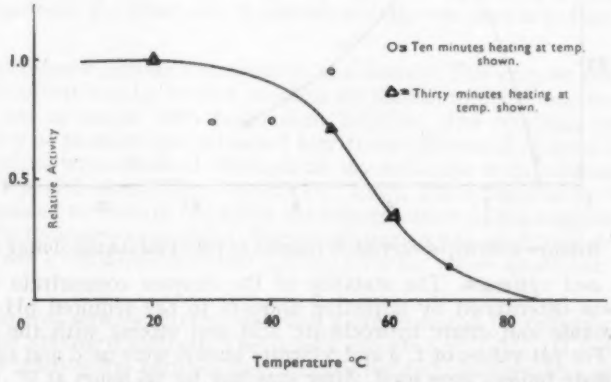

Fig. 1. Effect of different temperatures on enzyme stability.

Heat stability and temperature optimum. In testing for heat stability, aliquots of the enzyme concentrate at pH 5·2 were kept at different temperatures for 10 or 15 minutes, cooled and tested for cellulase activity, using the M/20 acetate buffer at pH 5·6. The results, given in Fig. 1, indicated that inactivation became marked above 50°. Somewhat erratic results were obtained for the short heating time at lower temperatures.

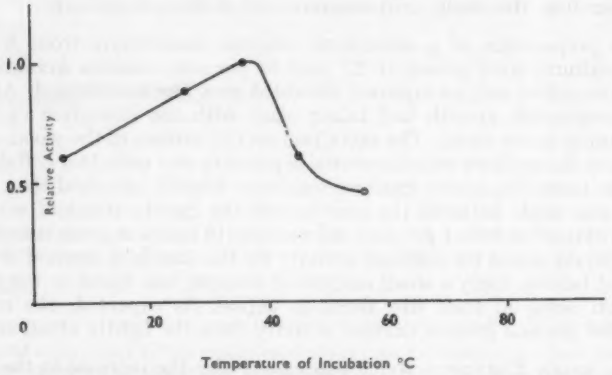

Fig. 2. Relative activity of enzyme in relation to temperature of incubation.

The temperature optimum was determined in M/20 acetate buffer at pH 5·6 by carrying out the cellulase assay at various temperatures and determining the temperature for optimal rate of hydrolysis. The results given in Fig. 2 indicate an optimum at about 37°. This represented an overall optimum, being the sum of the effects of temperature activation and destruction of the enzyme by heat. The unit for relative activity in Figs. 1 and 2 is equivalent to a difference in titre of 3·3 ml. of N/200 thiosulphate between the boiled and unboiled enzyme solutions, the titre for the former being 11·13 ml. of N/200 thiosulphate.

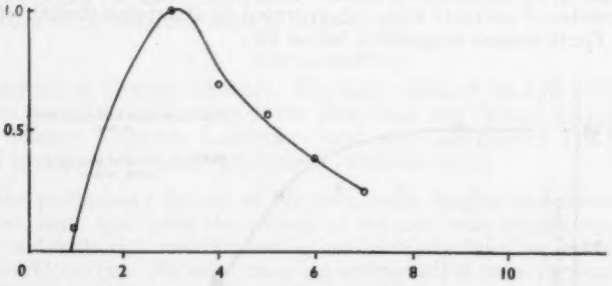

Fig. 3. Relative activity of enzyme in relation to pH of incubation during assay.

pH stability and optimum. The stability of the enzyme concentrate at different pH values was determined by adjusting aliquots to the required pH with dilute sodium hydroxide and dilute hydrochloric acid and mixing with the appropriate M/20 buffer. For pH values of 1, 3 and 5 citrate buffers were used and for higher pH values phosphate buffers were used. After standing for 66 hours at 9°, the pH was

again determined and readjusted to pH 5·6. The enzyme activity was then assayed at pH 5·6 by the standard method.

The pH values of the mixtures changed after 66 hours in spite of the buffer solutions. Table II records both the initial pH and the final pH of the mixtures, as well as the activities at these pH values. The optimum stability of the enzyme was found to lie between pH 3 and 5, while the enzyme was not very sensitive to pH values between 2 and 6. However, inactivation became pronounced above pH 6.

The results also indicate that the isoelectric point of the proteins in the enzyme concentrate was between pH 1 and 3 since the pH of the enzyme concentrate tended to rise or fall towards this region[7].

TABLE II

Effect of pH on stability of enzyme at 9°

Original pH	pH after 66 hours at 9°	Relative activity (pH 5·6)
1·0	1·1	0·6
3·0	2·9	1·0
5·0	4·9	1·0
7·0	6·4	0·7
9·0	7·3	0·6
11·0	7·8	0·5
12·0	8·4	0·3

The activity unit in Table II represents a difference of 2 ml. $^{N}/_{200}$ thiosulphate between boiled and unboiled enzyme solutions, the former being about 10 ml., representing considerable amounts of reducing substances in the enzyme concentrate apart from enzyme activity.

The pH optimum at about pH 3 (Fig. 3) was determined by measuring the relative activity at 36° of the enzyme concentrate, using buffers of different pH values in the standard assay method. The same unit of relative activity was used as in Figs. 1 and 2.

Relationship between enzyme concentration and activity. The enzyme concentrate at pH 3·5 was concentrated by further vacuum distillation to about one tenth of its initial volume and saturated with magnesium sulphate. The resulting precipitate was sucked as dry as possible and extracted four times with small aliquots of water. The filtered extracts were desalted through an ion-exchange resin column (1 part Deacidite E + 1 part Zeokarb 225; Permitit Co., Ltd.). The volume of the resulting solution was adjusted to contain ten times the concentration of the enzyme concentrate from the Bartholomew evaporator. The concentration was arbitrarily designated as 32, but there was a considerable loss of activity after the above treatment.

This solution was serially diluted and each dilution tested for cellulose activity at pH 3·2 by the standard method. The results recorded in Table III indicate an essentially linear relationship between enzyme activity and concentration at the low concentrations of enzyme used in the experiment, and using a large excess of cellulose.

TABLE III

Relationship between enzyme concentration and activity

RELATIVE ENZYME CONCENTRATION (X)	REDUCING SUGAR EQUIVALENTS AS MLS. OF $^{N}/_{200}$ THIOSULPHATE						Difference Calculated Δ_{2-1}
	Boiled			Unboiled			
	Found (Y_1)	Calculated ($\hat{Y}_1$)	Residual	Found (Y_2)	Calculated ($\hat{Y}_2$)	Residual	
32	9·73	9·7547	−0·0247	11·13	11·0998	+0·0302	1·35
16	5·22	5·1707	+0·0493	5·76	5·8782	−0·1182	0·71
8	2·83	2·8787	−0·0487	3·35	3·2674	+0·0826	0·39
4	1·87	1·7327	+0·1373	2·06	1·9620	+0·0980	0·23
2	1·12	1·1597	−0·0397	1·28	1·3093	−0·0293	0·15
1	0·80	0·8732	−0·0732	0·92	0·9830	−0·0630	0·11

The above conclusion was confirmed by statistical analysis. Since for the Shaffer and Hartmann sugar reagent, over a restricted range of sugar concentrations, there is a linear relationship between reducing sugar concentration and amount of thiosulphate used in the titration, plotting the value Y_1 against X must give a straight line. Hence this plot may be used to estimate the true residual experimental variance. In this case, for the boiled solution, the least squares straight line is found to be: $Y_1=0{\cdot}5867+0{\cdot}2865X$ giving calculated ordinates ($\hat{Y}_1$) and residuals as in Table III, and the estimated residual variance $=\hat{\sigma}_1^2=0{\cdot}007799$ based on 4 degrees of freedom.

The hypothesis concerning the "unboiled" values is that they also lie on a straight line subject to the same experimental (residual) error variance, σ_1^2. Fitting a least squares straight line through these observations, $Y_2=0{\cdot}6566+0{\cdot}3264X$ and the calculated ordinates ($\hat{Y}_2$) and residuals are as in Table III. The estimate of σ_1^2 based on these numbers is $\check{\sigma}_1^2=0{\cdot}009032$ based on 4 degrees of freedom.

On the above hypothesis the ratio $\frac{\check{\sigma}_1^2}{\hat{\sigma}_1^2}$ should be an F variable. The calculated value of this ratio is 1·158 and upon entering this value in the suitable F-table we find that it is not significant. The hypothesis is therefore acceptable. As a consequence the difference is also a straight line, *viz.* $\Delta_{2-1}=0{\cdot}0699+0{\cdot}0399X$ and values calculated from this question are shown in the last column of Table III. The straight line does not pass through the origin because the line connecting the amount of thiosulphate used with the Shaffer and Hartmann reagent and the reducing sugar concentration, does not pass through the origin.

Hydrolysis products of cellulose and cellobiose. A cellulase concentrate was prepared from the original enzyme concentrate by saturation with ammonium sulphate, centrifuging and suspending the resultant precipitate in distilled water. The residual ammonium sulphate was removed by dialysis.

Hydrolysis of cellobiose and cellulose was carried out under standard assay conditions at pH 3·2 and the resultant solutions analysed for sugars by paper chromatography (butanol-acetic acid-water; 4:1:5). The inorganic salts were first removed by pyridine extraction[3] and the spots detected with aniline hydrogen phthalate[2]. Glucose was the major product of hydrolysis of cellulose in vitro, a small quantity of cellobiose being simultaneously produced.

DISCUSSION

The response to hydrogen ions and temperature of the cellulase from *H. henningsii* (T.R.L. 24) is not the same as that of cellulases from other sources. However, there is insufficient evidence to decide whether this is due to actual differences in the enzymes or to differences in substrates, duration of assay and temperature of assay.

The formation of glucose as the major product of hydrolysis of cellulose in vitro is interesting in that it has been found by the author that *H. henningsii* is able to grow more vigorously on cellobiose as substrate than on glucose, which corresponds with results reported by Siu[13] on *Myrothecium verrucaria*. However, the small quantity of cellobiose found in the hydrolysis products indicates that cellobiose is probably an intermediate in the hydrolysis of cellulose to glucose. Considerable cellobiose activity is also present in the enzyme concentrate, thus preventing cellobiose accumulation.

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THE RAPID AND ACCURATE DETERMINATION OF SULPHUR IN PYRITES BY THE COMBUSTION METHOD

by

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OPSOMMING

'n Metode vir die vinnige en presiese bepaling van die swawelinhoud van piriet word aangegee. Die piriet, wat met kieselguhr gemeng word om sintering te verhoed, word in 'n stroom suurstof by ongeveer 950° gebrand. Die gasmengsel wat ontstaan word eers deur 'n neutrale waterstofperoksiedoplossing geborrel en gaan daarna deur 'n droë asbesfilter om die suur miswolk terug te hou. Die totale swawelsuur wat gevorm word, word met standaard boraksoplossing titreer. Vir 'n swawelinhoud van omtrent 40 *per cent.* is die standaardafwyking minder as 0·10.

SUMMARY

A method for the rapid and accurate determination of sulphur in pyrites is given. The pyrites, mixed with kieselguhr to prevent sintering, is burnt in a stream of oxygen at about 950°. The emergent gases are bubbled through neutral hydrogen peroxide solution, then through a dry asbestos filter to retain acid "mist" and the total sulphuric acid formed is titrated with standard borax solution. For a sulphur content of about 40 *per cent.* the standard deviation is less than 0·10.

The combustion methods described [1-10] for the determination of sulphur in pyrites, while considerably more rapid than the wet oxidation or fusion methods, suffer from disadvantages such as high combustion temperatures (sometimes up to 1450°), complicated apparatus or an empirical approach. During a visit to the Johannesburg Consolidated Investment Group Laboratory the author was shown a simple and rapid combustion method in which the pyrites was burnt in a stream of oxygen at about 950°, the products of combustion absorbed in dilute hydrogen peroxide and the sulphuric acid formed titrated. The results were, however, low by a constant factor. This paper describes a modification of this empirical method which gives quantitative recovery of sulphur.

EXPERIMENTAL

The apparatus used is shown in Fig. 1.

Procedure. 0·1 to 0·15 g.kieselguhr in an unglazed refractory boat was ignited at 900° for 2 hrs. and stored in a desiccator. The asbestos filters were prepared by packing dry asbestos into hollow glass stoppers, provided with holes of 1 mm. diameter, in such a way that it was possible to blow easily through them and were attached to the tube with rubber bands.

With the bubbler containing 5 inches of neutral "1·5 volume" hydrogen peroxide solution (prepared daily and neutralised if neccessary, using the mixed indicator prepared fortnightly from 0.125 methyl red and 0·083 g. methylene blue in 100 ml. isopropyl alcohol) connected to the combustion tube, the furnace was set at 940°. The boat containing the ignited kieselguhr was weighed after standing for 5 minutes in the balance case and the weighed amount (0·1 to 0·12 g.) of dry crushed pyrites added. After careful mixing of the contents with a platinum wire, the boat was

pushed into the silica tube up to the constriction, the oxygen immediately connected and adjusted to a flow of 2 l. per minute (a needle valve allowing rapid adjustment of the flow rate was essential) and combustion allowed to proceed for 10 minutes.

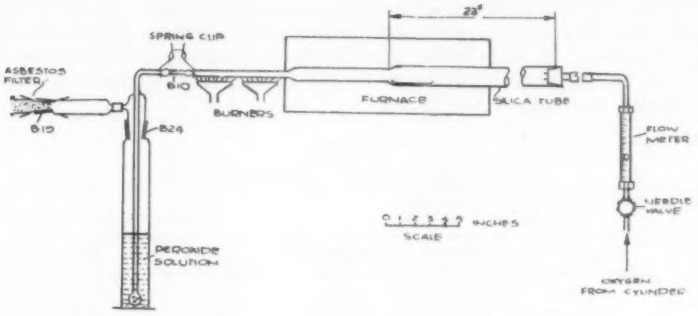

Fig. 1. Apparatus for the determination of sulphur by combustion.

The contents of the bubbler, filter and all connections were washed into a beaker and titrated with standard 0·1 N borax solution (mixed indicator as above; although this indicator is sensitive to carbon dioxide, it was found that this gas, if present, was effectively swept out by the oxygen stream. In any event, pyrites from South African gold mines contains little, if any, carbonaceous matter. The borax solution was stable for at least 4 months). It was essential to heat the portion of the tube projecting from the furnace to prevent retention of acid.

RESULTS AND DISCUSSION

The J.C.I. Group Laboratory procedure suffered from two main disadvantages, *viz.* loss of acid "mist" during combustion and retention of sulphur in the burnt residue.

Loss of acid "mist". Combustion of pyrites yields both sulphur dioxide and sulphur trioxide, the latter forming sulphuric acid mist which cannot be retained by the normal absorbing solutions. The use of an open measuring cylinder to absorb the products of combustion resulted in loss of mist and results were accordingly low; e.g., combustion analysis of one sample (A) gave 38·84, 38·59 and 38·63 *per cent.* sulphur, while analysis of the sample by the peroxide fusion method gave 39·41 *per cent.*

With practice, the filter shown in Fig. 1 could be prepared in such a way as to retain the mist quantitatively while allowing the oxygen to flow through it at the rate of 2 l. per minute. The mist retained by the asbestos corresponded to about 0·4 to 1·2 *per cent.* sulphur in the pyrites sample. The retention of sulphuric acid mist by sintered glass is standard practice [11, 12], but with this system it is usual to apply suction at the end of the bubbler, using air as the combustion medium. The asbestos filter was used since oxygen could not be blown through a sintered glass disc at a sufficient rate.

Sintering of samples during combustion. Some degree of sintering of the pyrites samples invariably occurred during combustion, the greater the sintering the lower the results, due, probably, to occlusion of sulphur compounds in the sintered globules. Sintering was dependent on the type of boat used and on the manner in which the pyrites was distributed along the bottom of the boat. Unglazed refractory boats were

found best; in smooth silica boats sintering was especially pronounced. Sintering was avoided to some extent by distributing the sample evenly in the boat before combustion. Despite this, however, the residue after combustion still contained the equivalent of 0·1 to 0·2 *per cent.* sulphur in the original pyrites. The sulphur was located almost entirely in the sintered globules with only trace amounts in the powdery fraction of the residue. By crushing the residues and reburning, however, practically all the sulphur was recovered. Results using this double combustion technique are shown in Table I; for comparison the analyses of the same pyrites samples by the sodium peroxide fusion method are also given.

TABLE I

	Combustion analysis			Na_2O_2 fusion analysis		
Pyrites sample	No. of determinations	Mean % S	Standard deviation	No. of determinations	Mean % S	Standard deviation
B	18	41·00	0·06	12	41·00	0·28
C	6	43·62	0·04	12	43·21	0·19
D	6	41·18	0·10	7	40·96	0·07

Sintering could be more satisfactorily avoided by mixing the pyrites with kieselguhr, previously ignited at 900°, with a platinum wire in the boat. No apparent sintering occurred in the combusted samples and the results were as good as those obtained by the double combustion method. The figures obtained by three operators are shown in Table II.

TABLE II

	Pyrites sample C			Pyrites sample D		
Operator	No. of determination	Mean % S	Standard deviation	No. of determination	Mean % S	Standard deviation
X	6	43·56	0·09	5	41·16	0·02
Y	6	43·59	0·09	6	41·18	0·07
Z	—			6	41·14	0·04

Other factors in the analysis. Variation of the oxygen flow from $1\frac{1}{2}$ to $2\frac{1}{2}$ l. per min. had little effect on the results (Table III) and a rate of 2 l. per min. was selected as standard. Variation of the temperature from 890° to 980° had no effect as shown by the figures in Table IV.

The pyrites samples used in this investigation were from three sources and had widely differing metallic impurity contents. In all cases the results obtained by combustion agreed well with those from peroxide fusion analyses. One of the advantages of the combustion method is that it measures "combustible sulphur" which is all that can profitably be used in the manufacture of sulphuric acid. In the case of the samples examined, the total sulphur and combustible sulphur were apparently identical. The combustion method is considerably more precise than the fusion method, as can be judged from the values for standard deviation given in the Tables, is manipulatively easier and approximately three times as fast. In addition there appears to be no operator bias in the method.

TABLE III

Effect of Oxygen Flow (Pyrites sample B)

Rate of O_2 flow (l./min.)	No. of determinations	Mean % S	Standard deviation
$1\frac{1}{2}$	5	41·03	0·10
2	6	40·97	0·13
$2\frac{1}{2}$	4	41·11	0·04

TABLE IV

Effect of Temperature (Pyrites sample A)

Temperature °C	No. of determinations	Mean % S	Standard deviation
890	6	39·42	0·08
980	11	39·44	0·08

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PREPARATION OF DOUBLE METAL SULPHIDES OF THE TYPE AB_2S_4. PART I. COMPOUNDS OF TITANIUM, GOLD AND ALUMINIUM

by

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OPSOMMING

Drie dubbelmetaal-sulfides, $CuTi_2S_4$, $TiZr_2S_4$, en $TiAl_2S_4$ is berei deur die samestellende elemente in stoigiometriese verhoudings in verseëlde geëvakueerde silikabuise te verhit. Hulle kristalliseer as spinelle met a=9·993, 10·26, en 9·945 Å. resp. Soortgelyke verhitting van ander mengsels van elemente met swael of H_2S en titanium, goud of aluminium het net binêre sulfides gegee.

SUMMARY

Three double metal sulphides, $CuTi_2S_4$, $TiZr_2S_4$, and $TiAl_2S_4$ have been prepared by heating the constituent elements in stoichiometric proportions in sealed evacuated silica tubes. They crystallised as spinels with a=9·993, 10·26, and 9·945 Å. respectively. Similar heating of other mixtures of elements with sulphur or H_2S and titanium, gold or aluminium, gave only binary sulphides.

Ternary compounds with the general formula AB_2X_4 usually crystallise in one of the following five structure types: spinel, $MgA1_2O_4$; chrysoberyl, $BeA1_2O_4$; K_2SO_4, K_2SO_4, related to chrysoberyl; phenacite, Be_2SiO_4; and K_2PtC1_4 [1]. The spinel structure is stable through a wide range of ionic sizes which makes it particularly important, a great variety of compounds crystallising in this form.

There are a great number of double metal oxides of the general formula AB_2O_4 with the spinel structure. A large proportion of them occur naturally as minerals and much work has also been done on their synthetic preparation [2]. Three classes have been found: (i) $A^{I}B^{III}_2O_4$; 2:3 Spinels with bivalent and tervalent cations, e.g. the mineral spinel $MgAl_2O_4$ and $MgFe_2O_4$. (ii) $A^{IV}B^{II}_2O_4$; 4:2 Spinels, e.g. Mg_2SnO_4, Co_2SnO_4 and $TiMg_2O_4$. (iii) $A^{VI}B^{I}_2O_4$; 6:1 Spinels, e.g. Li_2MoO_4.

The cubic unit cell of a "normal" spinel such as $ZnAl_2O_4$ contains eight molecules; the space group is Fd3m (Schoenfles symbol O^7_h). A 32-fold set of positions (32b sites) is occupied by oxygen ions, an 8-fold set of positions (8f, tetrahedral sites) is occupied by bivalent zinc ions, each of which is found to be surrounded tetrahedrally by four oxygen ions, and a 16-fold set of positions (16c, octahedral sites) is occupied by tervalent aluminium ions, each of which is then surrounded octahedrally by six oxygen ions.

In the "inversed" spinels, e.g. $MgFe_2O_4$, the cations are distributed differently over the same pattern of sites. Half of the tervalent ferric ions of magnesium ferrite occupy the tetrahedral sites, while the other half, together with all the bivalent magnesium ions, are distributed at random over the octahedral sites [3]. The tetrahedral sites of 4:2 inversed spinels are occupied by bivalent ions and the octahedral sites by a random arrangement of eight bivalent and eight quadrivalent ions. It is important that inversed spinels have identical lattice points occupied at random by two different ionic species, so that the structure has "variate atom equipoints".

Some spinels are ferromagnetic, e.g. magnetite, and magnesium, manganese and nickel ferrites. From the point of view of possible industrial applications and uses this is a most important property. It appears that the inverse structure is favourable and probably necessary for spinels to be ferromagnetic[2]. Electronic conductivity in magnetite has been studied by Verwey and de Boer[4]. When the lattice contains ions of the same element but of different valency in equivalent positions, electronic conductivity is favoured.

Since oxygen and sulphur are closely related chemically, it is not surprising that double metal sulphides exist as *thiospinels* in which sulphur appears in the place of oxygen in the oxide spinels. The ease of formation of the thiospinels would, however, be likely to be influenced largely by the fact that the ionic radius of sulphur is much larger than that of oxygen. Seleno- and tellurospinels have also been reported [5, 6] and the oxygen can also be replaced by the cyanide radical.

A list of some of the thiospinels which have been prepared synthetically is given in Table I.

TABLE I

Thiospinel	References	Remarks
Co_3S_4	7, 8, 9	
$CuCo_2S_4$	9	Doubtful
$FeCr_2S_4$	10, 11, 9	
$MnCr_2S_4$	12, 9	
$CdCr_2S_4$	12	See also 11
$ZnCr_2S_4$	13, 14, 9	See also 11
$HgCr_2S_4$	11	
$CoCr_2S_4$	11, 9	
$CuCr_2S_4$	9, 6	
Fe_3S_4	15	Queried by 7 Claimed as mineral, 17
Ni_3S_4, $FeNi_2S_4$	18	9 found tendency for Ni^{3+} compounds to crystallise ir hexagonal system.
CuV_2S_4	6	
MIn_2S_4, where M=Mg, Ca, Cd, Hg, Mn, Fe, Co, Ni.	19	
$ZnAl_2S_4$	5	

The work now reported was carried out with the aim of extending our knowledge of the preparation and crystal chemistry of double metal sulphides.

EXPERIMENTAL

For the preparation of double metal sulphides two of the methods described in the literature were used, namely (i) heating a mixture of the elements or binary sulphides together with sulphur, if necessary, in the calculated (stoichiometric) proportions in sealed evacuated silica tubes, and (ii) heating a mixture of the elements in the calculated proportions in an atmosphere of hydrogen sulphide.

Method (i). Calculated quantities of the substances in question (total weight 0·5 g.) were thoroughly mixed and compressed into shining metal-like pellets. The latter, broken into two or three fragments were introduced into "Vitreosil" tubes about 10 cm. long with an internal diameter of 5 mm. The open end of a tube was fused to a "lead" attached to a ground joint, making a constriction at the junction. The whole was then evacuated to a pressure of about 0·0005 mm. Hg and the tube sealed off at the constriction.

The sealed evacuated tubes were heated at between 800 and 1,000° in a tubular furnace for periods ranging from 2 hours to a few days. After the tubes had cooled off completely in the furnace, they were carefully broken, care being taken not to contaminate the products with splinters from the "Vitreosil" tubes. In almost all cases the tubes smelt strongly of hydrogen sulphide after they had been opened, probably due to moisture absorbed by the reaction mixture, which could not be completely removed under vacuum.

Method (*ii*). The reaction mixture was placed in a small porcelain boat which was pushed into a porcelain tube passing through the tubular furnace. Purified and dried hydrogen sulphide was passed over the reaction mixture and led out through a U-tube filled with calcium chloride and finally through a bubbler. Before heating up the furnace all air was displaced from the apparatus with hydrogen sulphide and the gas current then adjusted to a steady rate of about one bubble every four seconds. This rate of gas flow was maintained throughout the period of heating (2 to 9 hours at 800 to 900°).

X-ray Investigation. The products were ground to a fine powder in an agate mortar and specimens placed in Lindemann glass capillaries or mounted with Canada Balsam on fibres drawn from Pyrex glass. These were then fitted and carefully centred in 9 cm. X-ray powder cameras. X-rays were generated by a Newton Victor "Raymax" set with a demountable tube and a series of interchangeable targets. The spacings of the lines on the films obtained were measured with a Hilger film measuring rule.

In a few cases the relative intensities of the arcs on the film were measured by means of a Hilger and Watts microphotometer connected to a sensitive mirror galvanometer. Galvanometer readings were taken at 0·5 mm. intervals on both sides of and across the arcs. Logarithms of the galvanometer readings were plotted against the distance traversed. The relative intensities were obtained by cutting out the areas between the peaks and the extrapolated curves representing the background in the vicinity of any particular arc, and weighing the cuttings so obtained.

Known compounds in mixtures were identified mainly with the aid of the A.S.T.M. Index [20].

RESULTS

The sulphides of titanium. Although these compounds have been fully investigated [21, 22], an attempt was made to prepare Ti_3S_4 as a spinel analogous to Co_3S_4 and Ni_3S_4, by method (i) ($5\frac{1}{2}$ hours at 940°). The X-ray pattern of the shiny black product showed that combination between titanium and sulphur had taken place, but the product was not a spinel.

Systems containing titanium. Titanium in combination with another multivalent metal leads to the double metal sulphides MTi_2S_4 and TiM_2S_4 being either 2:3 or 4:2 spinels.

Ti-Cu-S. A mixture of the elements was made up in the proportions for $CuTi_2S_4$, compressed and heated under vacuum at 900° for 3 hours. A dark grey powdery substance was obtained. The X-ray pattern had 22 measurable lines, all of which could be indexed for a spinel. There were no indications of copper or titanium sulphides. A mixture with a total weight of 1 g. instead of the usual 0·5 g., was then heated for a week at 860°. The product had the same appearance as before and the X-ray pattern with 33 lines included all the lines of the previous pattern; all could be indexed for a spinel structure. The pattern is given in Table II, and was that of a spinel with a= 9·993 Å. The intensities given up to index 533 were determined by microphotometer and the rest estimated. Copper radiation and a nickel filter were used.

TABLE II

Index	Intensity	Spacing d (Å)	$\sin^2\theta$ Expt.	$\sin^2\theta$ Calc.
111	0·68	5·98	0·0166	0·0179
220	3·45	3·588	0·0461	0·0476
311	7·38	3·046	0·0639	0·0655
222	0·75	2·910	0·0700	0·0714
400	5·76	2·513	0·0939	0·0952
422	3·13	2·043	0·1421	0·1428
511	3·49	1·927	0·1596	0·1607
440	10·00	1.769	0·1894	0·1904
620	1·90	1·580	0·2373	0·2380
533	3·40	1·523	0·2553	0·2556
444	4	1·442	0·2848	0·2856
642	3	1·336	0·3323	0·3332
731	5	1·300	0·3505	0·3515
800	5	1·249	0·3798	0·3808
822	1	1·177	0·4282	0·4284
751	5	1·153	0·4463	0·4443
840	5	1·116	0·4736	0·4760
931	4	1·047	0·5413	0·5415
844	9	1·019	0·5718	0·5712
862	1	0·979	0·6188	0·6188
773	4	0·965	0·6371	0·6367
10, 42	1	0·911	0·7149	0·7140
775	1	0·899	0·7335	0·7319
880	7	0·883	0·7618	0·7616
866	1	0·856	0·8091	0·8092
973	1	0·848	0·8259	0·8271
884	7	0·832	0·8564	0·8568
10, 64	2	0·810	0·9039	0·9044
975	4	0·802	0·9221	0·9223
12, 40	8	0·790	0·9517	0·9520

TABLE III

Index	Intensity	Spacing d (Å)	$\sin^2\theta$ Expt.	$\sin^2\theta$ Calc.
111	5	6·14	0·0157	0·0169
311	6	3·129	0·0606	0·0620
222	5	2·995	0·0661	0·0677
——	1	2·873	0·0718	
400	10	2·583	0·0889	0·0902
331	5	2·371	0·1049	0·1071
511	4	1·987	0·1502	0·1528
440	10	1·819	0·1793	0·1805
531	4	1·738	0·1963	0·1974
622	5	1·549	0·2470	0·2482
444	7	1·482	0·2699	0·2707
711	3	1·437	0·2871	0·2876
731	4	1·336	0·3323	0·3328
800	5	1·282	0·3605	0·3610
662	2	1·179	0·4268	0·4286
840	8	1·146	0·4515	0·4512
844	7	1·046	0·5427	0·5414
933	4	0·9050	0·5580	0·5584
884	6	0·8529	0·8157	0·8122
12, 40	5	0·8094	0·9055	0·9024

Preparation of $TiCu_2S_4$ was also attempted (one day at 840°). The X-ray pattern of the product gave 36 lines. Formation of the spinel $CuTi_2S_4$ definitely took place,

as 19 lines could be attributed to it, all the strongest lines being present. The remaining lines corresponded mainly to CuS, while faint indications of TiS_2 were present.

Zr-Ti-S. Treatment by method (i) of a mixture of the constituent elements in proportion for $TiZr_2S_4$ (overnight at 800°), gave a dark brown, almost black, material. In the X-ray pattern of 20 lines, only one, of estimated intensity 1, could not be indexed for spinel structure. The pattern corresponded to a spinel with $a=10{\cdot}26$Å. The intensities were estimated. Copper radiation and a nickel filter were used. Details are given in Table III.

A mixture to give $ZrTi_2S_4$, heated for an hour at 1050°, gave partial formation of the spinel $TiZr_2S_4$ and indications of ZrS_2 and TiS.

Other systems containing Ti. Table IV summarises the results of other attempts at forming spinels by method (i).

TABLE IV

Attempted Compound	Period of Heating	Temp. °C	Products Identified
$NiTi_2S_4$	3 hours	900	Ti_2S_3, NiS.
$TiNi_2S_4$	overnight	800	TiS_2, NiS, NiS_2.
$PbTi_2S_4$	5 hours	900	Binary sulphides.
$TiPb_2S_4$	1 day	840	Binary sulphides.
$MnTi_2S_4$	5 hours	900	Ti_2S_3, MnS.
$TiMn_2S_4$	1 day	580	
	and 1 day	830	TiS_2, MnS.
$HgTi_2S_4$	3 hours	800	Ti_2S_3, HgS.
$TiHg_2S_4$	3½ hours	860	TiS_2, HgS.
$SnTi_2S_4$	3½ hours	900	TiS_2, SnS, SnS_2, Ti, Sn.
$FeTi_2S_4$	3½ hours	900	TiS_2, FeS (pyrrhotite).
$TiFe_2S_4$	overnight	550	TiS_2, FeS (pyrrhotite),
	and then 12 hours	830	FeS (marcasite).
WTi_2S_4	5 hours	900	WS_2, various Ti-sulphides.
$ZnTi_2S_4$	6 hours	900	Ti_2S_3, $\alpha-ZnS$, $\beta-ZnS$.
$TiZn_2S_4$	overnight	900	TiS_2, $\alpha-ZnS$.
$CdTi_2S_4$	3 hours	900	Ti_2S_3, $\alpha-CdS$.
$TiCd_2S_4$	overnight	850	TiS_2, $\alpha-CdS$.
$MoTi_2S_4$	3½ hours	900	MoS_2, TiS, Ti.
$MgTi_2S_4$	3 hours	900	MgS, Ti_2S_3, TiS_2.
$TiMg_2S_4$	overnight	800	MgS, TiS_2.
$CrTi_2S_4$	1 hour	1,050	Ti_2S_3, CrS.
$TiCr_2S_4$	overnight	840	TiS_2, CrS.
$CoTi_2S_4$	3 hours	900	Binary sulphides.
$TiCo_2S_4$	2 days	800	TiS_2, Co_2S_4, CoS, Co_4S_3.
$TiSb_2S_4$	overnight	800	Sb_2S_3, Ti-sulphides.

Systems containing gold. Attempted preparation of double metal sulphides containing gold from a mixture of the elements in the proportions for MAu_2S_4 was carried out in a number of cases by method (i). Heating took place for periods between 3 and 5½ hours at 900-950°. In all cases except one, gold remained uncombined in the product, together with a simple sulphide (or sulphides) of the other metal. Preparation of the following was attempted:—$FeAu_2S_4$, $MgAu_2S_4$, $ZnAu_2S_4$, $PbAu_2S_4$, $TiAu_2S_4$, $SnAu_2S_4$, $CuAu_2S_4$, $MnAu_2S_4$ and $NiAu_2S_4$. With a mixture for WAu_2S_4, it was found that gold had entered into combination with the sulphur, but a double metal sulphide had not formed.

Systems containing aluminium. Ti-Al-S. The calculated amounts of titanium, aluminium and sulphur powder were mixed in proportions for $TiAl_2S_4$ and treated

by method (i) (7 hours at 880°) to give a pitch black product. Except for 2 lines, both of estimated intensity 1, the whole X-ray pattern of 23 lines could be indexed for spinel structure. The preparation was repeated with a larger quantity (1 g.) (one week at 860°). The X-ray pattern of the product had 27 lines, including 20 lines of the previous pattern. The (331) and 860) reflections of the previous pattern were absent, as well as one of the lines which did not fit for spinel structure. In the new pattern one line, also present in the previous pattern and of estimated intensity 1, could not be indexed for spinel structure. Details are given in Table V. The pattern corresponded to that of a spinel with $a=9{\cdot}945$ Å. The intensities up to that of reflection (440) were determined by microphotometer, the rest were estimated (copper radiation with a nickel filter).

TABLE V

Index	Intensity	Spacing d (Å)	$\sin^2\theta$ Expt.	$\sin^2\theta$ Calc.
111	0·46	5·90	0·0170	0·0180
220	0·45	3·531	0·0476	0·0481
311	1·97	3·035	0·0644	0·0661
222	1·89	2·883	0·0714	0·0721
400	5·72	2·449	0·0950	0·0961
—	1	2·097	0·1349	
422	1·33	2·007	0·1470	0·1442
511	3·49	1·919	0·1609	0·1622
440	10·00	1·757	0·1922	0·1923
531	2	1·682	0·2096	0·2103
622	5	1·499	0·2637	0·2644
444	7	1·431	0·2895	0·2884
711	2	1·396	0·3038	0·3064
731	4	1·292	0·3546	0·3545
800	7	1·241	0·3849	0·3845
662	3	1·143	0·4534	0·4566
840	8	1·113	0·4791	0·4808
931	3	1·042	0·5465	0·5467
844	5	1·014	0·5768	0·5768
773	3	0·961	0·6430	0·6429
880	5	0·880	0·7669	0·7690
955	3	0·870	0·7835	0·7870
10, 62	2	0·840	0·8411	0·8411
884	4	0·829	0·8641	0·8651
12, 40	7	0·786	0·9599	0·9613

Other systems containing aluminium. With periods of heating from 3 to 16 hours at temperatures from 840 to 900°, method (i) was used in attempts to prepare the following:—$MnAl_2S_4$, $SnAl_2S_4$, $CuAl_2S_4$, $FeAl_2S_4$, $CoAl_2S_4$, $MgAl_2S_4$, $NiAl_2S_4$. Simple sulphides but no double metal sulphides of the metals were obtained. Method (ii) was tried without success for the preparation of $FeAl_2S_4$, $CuAl_2S_4$, $SnAl_2S_4$ and $MnAl_2S_4$.

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